CULTIVATION OF THE NEW ZEALAND GEODUCK CLAM, *Panopea zelandica*

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Abstract

The geoduck *Panopea zelandica* has been signalled as a new emerging species for aquaculture in New Zealand. To pave the way for the establishment of a geoduck aquaculture industry, information on how to grow this species over its life cycle needs to be determined. The aim of this study was to identify conditions that optimize *P. zelandica* broodstock conditioning, fertilization, larval growth and metamorphosis, and juvenile and young adult growth.

P. zelandica broodstock were conditioned within a combination of three water temperatures (7–8, 11–12, and 16–17°C) and three feeding rations (10,000, 50,000, and 100,000 cells mL^{-1} of *Chaetoceros muelleri* and *Tisochrysis lutea*, 1:1 cell counts) for 73 days. After conditioning, similar percent matured and dry condition index values were observed on geoducks among temperatures. However, significantly higher dry gonadosomatic indices (GSI_{dw}) were recorded at 8 and 12°C. Although no difference was detected in the percentage of spawned individuals and connective tissue occupation indices, a higher percent of matured individuals was recorded when fed 10,000 and 50,000 cells mL^{-1}. Glycogen, protein, and lipid analyses indicated that geoducks within all treatments achieved a positive energy balance, except for those in the treatments combining the highest temperature and lowest feeding ration. Comparisons of fatty acid profiles of animals among treatments and with the reference group (pond water conditioned) revealed that eicosapentaenoic (EPA, C20:5n-3), docosahexaenoic (DHA, C22:6n-3) and arachidonic (ARA, C20:4n-6) fatty acids were important contributors to gametogenic development for geoduck conditioning.

The development of *P. zelandica* embryos at 15°C and 35 ppt and the optimal sperm:egg ratios for fertilization under hatchery conditions were investigated. Fertilization was conducted at sperm:egg ratios of: 50:1, 100:1, 500:1, 1000:1, and 10,000:1 with a sperm-egg contact time of 40 min. The optimal sperm:egg ratio was determined to be < 500:1 and the normal embryo yield at 3 and 18 h post-fertilization ranged from 83-96%. *P. zelandica* eggs (~ 80 µm diameter) developed the first and second polar bodies within 15 - 20 and 50 - 55 min post-fertilization, respectively. The blastula appeared at ~ 8 hpf, including the X^R and X^L cells and the presumptive shell field depression. Gastrulation occurred at 12 - 18 hpf with organic material shell apparent at the shell field depression. The mid-stage trochophore, which appeared at around 35 hpf had an apical plate with an apical tuft. The shell field spread to form the periostracum, which expanded and folded into right and left segments covering the late trochophore. The early D-stage veliger appeared at 45 hpf with the soft body being enclosed by two valves and the appearance of the velum.

The physiological, morphological, and behavioral characteristics throughout the larval developmental process were determined for *P. zelandica* larvae, which were reared in a flow-through system at 17°C and 35 ppt. The initial veliger stocking densities ranged from 50 - 200 larvae mL^{-1} and geoduck larvae were fed continuously with *Tisochrysis lutea* and *Chaetoceros calcitrans* at residual algal levels of 20,000 to 80,000 cells mL^{-1} in three rearing batches. The larval development took 16 - 19 days from first D-veliger and metamorphosis occurred across a wide size range (300 - 375 µm shell length). The increase in shell length was linear over time and correlated with the deposition of striae in the prodissoconch II. The ingestion rate followed a power function with time and was closely correlated with the development of the alimentary system. Rearing

with an initial stocking density of 100 larvae mL^{-1} and residual algal background of 20,000 cells mL^{-1} resulted in about 76% survival and 15 µm day^{-1} growth rate.

The metamorphic induction of larval *P. zelandica* was tested with different neuroactive compounds. Two batches of competent hatchery-reared larvae were exposed to acetylcholine chloride, epinephrine hydrochloride, and excess potassium ions in the form of KCl and K_2SO_4 for 3 and 24 h. None of the tested chemicals increased the proportion of metamorphosed geoducks, and in some cases the chemical inhibited metamorphosis and caused significant mortality, despite having been used extensively with other species, such as mussels and oysters.

The allometric coefficients (*β*) of respiration rate RR and clearance rate CR in *P. zelandica* were 0.73±0.03 and 0.62±0.07, respectively.

P. zelandica juvenile and young adults were acutely thermally challanged at five different temperatures representative of potential farming conditions (8, 11, 15, 19, and 23°C). Their aerobic scope for activity and clearance rates were determined at all temperatures. Comparisons of aerobic scope for activity and clearance rates between size classes revealed that juvenile geoducks had a narrower thermal optimum than young adults (15 – 19°C *versus* 11 – 19°C, respectively). Temperatures higher than 19°C resulted in a reduction of aerobic scope for activity and clearance rate for both juvenile and young adults, which may lead to reduced performance and elevated mortality.

P. zelandica juvenile and young adults were exposed to normoxia, mild hypoxia, and severe hypoxia. The respiration, aerobic scope, critical oxygen partial pressure (P_cO_2), and oxygen regulation capacity in two size classes of fed and starved animals were determined. The P_cO_2 was determined to be ~4 kPa for

all geoduck groups. The respiration rates of fed small geoducks decreased significantly from normoxia (16.7 - ~21 kPa) to mild hypoxia (P_cO_2 - 16.7 kPa). Conversely, the respiration rates of starved geoducks from both size classes, and large fed geoducks were maintained at a constant level when exposed to the same change in oxygen concentration. However, all geoducks experienced decreased respiration rates during severe hypoxia (0 kPa - P_cO_2). In addition, overall oxyregulatory capacity, assessed using a regulation index, was affected by size rather than by nutritional stress. Large geoducks maintain oxygen consumption across an oxygen gradient more effectively than small geoducks. Also, the aerobic scope of small geoducks decreased significantly with declining PO_2, while large geoducks maintained their aerobic scope under hypoxia.

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Attestation of Authorship

“I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.”

Authorship Agreement Form

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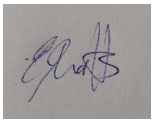

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CHAPTER 1 - Introduction

1.1 General Introduction

Geoduck clams (*Panopea* spp.) are the world's largest burrowing clams and were cosmopolitan during the Cretaceous period. Their fossil record has been found over North America, South America, Africa, Europe, Asia, Pacific Islands and Antarctica (www.fossilworks.org). However, living geoducks are currently found only in a few countries, including the United States of America (USA), Canada, Mexico, Argentina, Japan, Korea, Italy, Spain, and New Zealand (Leyva-Valencia et al., 2015; Scotti et al., 2011). The live weight of a geoduck can attain a maximum of 0.5 to 3.25 kg individual^{-1} (Lee et al., 1998; Goodwin and Pease, 1989), of which their siphons and mantle are responsible for about 42% (Oliveira et al., 2011). Geoducks dig deep into the seabed, extending their siphons to the sediment surface for ventilation and respiration. Their siphons can extend up to four times longer than their shell length, which is up to 80 – 100 cm (Goodwin and Pease, 1989). Unlike other bivalves, the siphons are too big to retract into the shells, and once mature they are often unable to re-bury if disturbed. This rather unique lifestyle may support their extremely long lifespans, which can be up to 168 years (Orensanz et al., 2004), without having lost their fertility (Goodwin and Pease, 1989). Due to long life and large size geoducks became an important role in seabed ecology (Norkko et al., 2013). Despite being incredibly long-lived and fertile, most populations of geoduck are small, and their distributions patchy, with relatively low recruitment (Campbell et al., 2004; Gribben et al., 2004b; Morsan et al., 2010). Thus, they may be a vulnerable species within an ecosystem under climate change pressures (Clark et al., 2013; Norkko et al., 2013).

Besides having an important ecological role, geoducks are considered as a luxury seafood. Geoducks have been highly esteemed in Asian seafood markets due to their huge phallic looking siphons. Additional contributing characteristics to their high values are the sweet taste and the crunchy cucumber-like texture of the siphons (GSGislason & Associates Ltd., 2012). Similar to the scallop adductor muscle (Shumway and Cembella, 1993; DeGrasse et al., 2014), geoducks siphons do not contain paralytic shellfish poisoning despite the appearance of toxic algae (Curtis et al., 2000). Thus, geoducks can be harvested and sold year around. They are mainly served as hot pot and quick fry meals, but also sashimi, sushi, sauté, chowder, ceviche, and vongole (Cap Log Group, 2013; GSGislason & Associates Ltd., 2012). Markets for geoducks are mainly in China, Hong Kong, and Taiwan, but also in Japan, South Korea, Singapore, Malaysia, Vietnam, Portugal, Canada, and USA (Cap Log Group, 2013). Geoducks are currently sold at $NZD 60-100 kg^{-1} in Asian markets, and first grade animals are served in high-end restaurants at $NZD400 $individual^{-1}$ (GSGislason & Associates Ltd. 2012; Morgan, 2015). Their high prices have resulted in high landed value of $25 - $30 NZD kg^{-1} (Shamshak and King, 2015).

Due to their high value and demand, the geoduck fishery and aquaculture have thus become lucrative industries. Wild Pacific geoducks (*Panopea generosa*) are mainly harvested in Washington and Alaska (USA), British Columbia (Canada), and Baja California (Mexico), and the endemic New Zealand species (*Panopea zelandica*) is also harvested in Golden Bay (New Zealand). Geoducks were harvested first in USA and Canada with the production peak of 7,000 tons in 1986. However, the production was reduced to less than 4,000 tons during 1992-2001 (Shamshak and King, 2015). The Mexican geoduck

fishery was established later in 2002. Geoduck production then increased from about 4000 tons in 2002 to 6,000 tons in 2009, but then decreased to 5000 tons in 2012 (Samshak and King, 2015). In contrast to the decline in production, the US export values increased from USD$40 million to over USD$60 million during 2009-2012 (Shamshak and King, 2015), showing that the demand kept increasing. Thus, the decline in production was not due to demand but harvested wild yield.

The yield of wild geoducks is limited by natural recruitment, which is low, temporally and spatially variable, and unpredictable (Campbell et al., 2004; Valero et al., 2004). Consequently, high fishing rates threaten the viability of the resource unless a suitable management action is taken (Aragón-Noriega et al., 2012). Fisheries regulations have been implemented to limit harvest to the exploitation rate of 1.7-2.7% in order to maintain the sustainability of industry in USA and Canada (Zhang and Hand, 2006). Similarly, although the production of New Zealand geoduck is still small, a quota system with a maximum total allowable catch was applied early in the fishery due to the small wild populations (Gribben and Heasman, 2015).

Due to the limit on fisheries, geoduck aquaculture has become a very desirable alternative to meet the demands and ensure the sustainability of the industry. Geoduck aquaculture was first established in the USA in the 1970s and then in Canada in the 1990s (Feldman et al., 2004). Cultivated geoduck production increased significantly from 2002 to 2008, and has been maintained at this level until 2012 (Shamshak and King, 2015). The cultivated geoduck production is limited due to technological bottlenecks and regulations rather than resources. Unlike most other cultured bivalves, wild spat geoducks cannot be caught because the adult populations distribute heterogeneously, and the spat do not

permanently attach or cement to substrates, but bury in the sand. Hence, the geoduck aquaculture industry must rely on hatchery production for seed. In addition, since geoducks require burrowing substrates and long grow-out times, the development of effective grow-out technology including site selection, growing systems, and harvesting methods to maximize the cost-effectiveness is essential.

The New Zealand aquaculture sector has to date been focusing on only three main species, primarily for export: Chinook salmon (*Oncorhynchus tshawytscha*), Pacific oyster (*Crassostrea gigas*), and green lipped mussel (*Perna canaliculus*). Exported volumes of these species in 2011 were mussel 38,143 tons, salmon 5,166 tons, and oyster 1,667 tons, generating an export revenue of NZ$ 218.1, 63.4, and 16.6 million, respectively, in 2011. The goal of the New Zealand aquaculture sector and the Ministry of Primary Industries is to achieve $NZD 1 billion in sales by 2025 (Carter, 2012). To achieve this goal, one strategy is to add values to those existing cultured species. Another strategy is to make viable the aquaculture of new species that are native or endemic, sustainable, marketable and high value. The three new target species identified for development are scallop (*Pecten novaezelandiae*), flat oyster (*Ostrea chilensis)*, and New Zealand geoduck (*P. zelandica*). Similar to *P. generosa*, the heterogeneous distribution and variable recruitment of *P. zelandica* make hatchery production the only viable option for seed procurement. However, very little scientific information is available regarding the biology, ecology, and cultivation conditions for the New Zealand geoduck.

1.2 Literature review

1.2.1 Current status of geoduck hatchery knowledge and techniques

Broodstock conditioning

Temperature and food are among the main external factors that control the rate of gonad maturation in marine bivalves (Utting and Millican, 1997). Temperature is a factor well-known to influence the reproductive biology of bivalves (Bayne et al., 1976; Loosanoff, 1937), including geoducks. Gribben et al. (2004a) demonstrated that although gametogenesis in geographically dispersed *P. zelandica* populations began in late autumn, spawning began during spring in one location (Kennedy Bay) and during late summer in another (Shelly Bay). However, temperature appeared to be the defining factor as both populations spawned when the temperature reached 15°C (Gribben et al., 2004a).

In a related species, *P. generosa*, animals collected from the wild and maintained at 10°C in flowing seawater for one month produced serial weekly spawning for four months (Beattie, 1994). Adopting this research, both Taylor Shellfish Hatchery (Washington, USA) and Nova Harvest Ltd. (British Columbia, Canada) maintained the wild collected *P. generosa* at ambient temperature (10-12°C) for several weeks before spawning attempts (Feldman et al., 2004; Dodd, 2014), although no other temperatures were trialed for comparison. Recently, Marshall et al. (2012) examined the effect of various temperatures on the reproductive development of *P. generosa*. *P. generosa* that were held at 7 and 11°C became mature faster than those at 15 and 19°C. Also, higher percentages of mature *P. generosa* were found at 7 and 11°C than at 19°C. In addition, the number of oocytes follicle^{-1} was higher at 7 and 11°C than at 15 and 19°C. Geoducks in the 7°C treatment had a significantly higher

gonadosomatic index than those in the 11°C treatment. However, there was no difference among groups held at 11, 15, and 19°C. When being induced to spawn, more geoducks at 11°C spawned at week 15-17 than at the other temperatures. It was noteworthy that gonads of *P. generosa* in the 19°C treatment degenerated, with 0 oocytes per follicle, and connective tissue occupied 90% of the gonad after 113 days. Thus, Marshall et al. (2012) recommended to condition *P. generosa* at 11°C so that the reproductive output could be maximized. In addition, a temperature of 7°C could be used to hold ripe *P. generosa* without spontaneous spawning. Such information suggests that *P. zelandica* might be induced to mature at temperatures between 7 and 19°C (Beattie, 1994; Feldman et al., 2004; Gribben et al., 2004; Marshall et al., 2012; Dodd, 2014).

Even if organisms are held at a favorable temperature they are exposed to poor food quality and/or quantity, their gonad maturation will be hindered (Utting and Millican, 1997). There has been only one published study examining the effect of different rations on the reproductive development of any geoduck species so far. In Marshall et al. (2014), *P. generosa* were fed *Tisochrysis lutea* and *Chaetoceros muelleri* (50:50 by cell count) at various rations of 0.8×10^9, 2.4×10^9, 4.0×10^9, 5.6×10^9, 7.2×10^9, and 10.0×10^9 cells geoduck^{-1} d^{-1}. The 10.0×10^9 cells geoduck^{-1} d^{-1} ration unexpectedly caused 25% moratlity within 3 d. Also, ration surprisingly did not significantly affect the reproductive indices (i.e. gonadosomatic index, connective tissue occupation index, and oocyte diameter). However, geoducks fed at 7.2×10^9 cells geoduck^{-1} d^{-1} not only had lower numbers of oocytes per unit area as well as levels of sperm occupation than those fed at lower rations, but also spontaneously spawned, which would reduce the reliability for hatchery operators. Thus, Marshall et al. (2014)

recommended that rations of 4.0×10^9 and 5.6×10^9 cells geoduck^{-1} d^{-1} would be adequate for *P. generosa* broodstock conditioning.

Manipulation of temperature and feeding ratios are key for a hatchery to control production across seasons by increasing the gametogenesis rate, maintaining condition index and shortening the conditioning period – as seen in *Tapes philippinarum* (Mann, 1979), and *Crassostrea gigas* (Chávez-Villalba et al., 2002). However, optimal temperature and feeding ratio for broodstock conditioning in *P*. *zelandica* has been unknown to date.

Spawning and fertilization

Geoducks can be induced to spawn by either thermal shock with food and sperm (Goodwin et al., 1979) or by serotonin injection (Gribben et al., 2014). Gametes of geoducks can also be collected by stripping (Gribben and Hay, 2003).

Although hatchery cultured geoduck *P. generosa* spat are now produced commercially in USA and Canada, information on sperm:egg ratios has rarely been released. In a study of producing triploid *P. generosa*, Vadopalas and Davis (2004) used the sperm:egg ratio of 40:1. Recently, Gribben et al. (2014) reported an optimum density of 100-10,000 sperm µL^{-1} for *P. zelandica,* with observations of high levels of polyspermy at higher sperm densities. However, the optimal sperm:egg ratio is also dependent on other factors, such as temperature, gamete age, and fertilization contact time (Gribben et al. 2014; Levitan, 2006; Stephano and Gould, 1988). Recommendations by Gribben et al. (2014) may not be the most applicable for hatchery practice. Therefore, it is necessary to develop optimal fertilization practices for hatchery production.

Embryo incubation

To date there has been only one published study of geoduck embryo incubation. Goodwin (1973) determined that salinity 27.5 – 32.5 ppt and temperature 6 - 16°C were the optimum conditions for embryo incubation in *P. generosa*. Knowledge of embryo development is very important for hatchery culture of bivalves. Bivalve embryogenesis has two notable features that relate to the organ development and shell formation of early larvae (Kin et al., 2009). The cleavage pattern feature determines the normal development of embryos, and consequently the normal development of organs, such as the velum, mouth, apical tuft and stomach in D-larvae (Hashimoto et al., 2014). However, there has been little study of geoduck embryological development.

Larval rearing

At present, the information available on geoduck larval rearing is also very limited. Early work on larval *P*. *generosa* development suggests that a stocking density of 3 larvae mL^{-1} is superior to 4–10 larvae mL^{-1} (Goodwin et al., 1979). Shaul (1981) suggested to rear *P. generosa* larvae at low density with an algal density of 30,000 to 50,000 cells mL^{-1}. However, their culture methods were borrowed from guidelines for other species that may not be optimal for geoduck. More recently, Marshall et al. (2014) examined the interactive effects of stocking densities (2, 5, or 10 larvae mL^{-1}) and feeding rations (5×10^3, 20×10^3, 40×10^3, 100×10^3 cells $larva^{-1}$ d^{-1}) on growth and survival of *P. generosa* larvae fed *T. lutea*. Percent survival over 23 d ranged from 7% to 56% and generally decreased with increasing stocking density. Marshall et al. (2014) showed that with respect to efficient use of space and feed, *P*. *generosa* larvae had the best combination of growth and survival with the lowest required amount of algae per larva at a density of 10 larvae mL^{-1} and a feeding ratio of 5×10^3 cells $larva^{-1}$ d^{-1}.

In general, survival rates of *P*. *generosa* were highest at a larval density of 2 larvae mL^{-1}, which indicates that reducing larval density as the larvae exceed 160 µm in shell length may be advisable (Marshall et al., 2014). The ingestion rate of *P. generosa* increased with increasing feed ration from 5,000 to 100,000 cells $larvae^{-1}$ day^{-1} (Marshall et al., 2014). In a similar study, Ferreira-Arrieta et al. (2015) demonstrated that the ingestion rate of *P. globosa* increased with an increase in algal concentration from 50,000 to 100,000 cells mL^{-1}, but reached the plateau between 100,000 to 300,000 cells mL^{-1}. However, all of these previous studies have used static systems to rear larval geoducks. To meet the demand of seed production, hatcheries can increase the scale of larval production either by increasing the volume of static tanks at low stocking densities or by increasing stocking densities using flow-through culture systems (Supan, 2014). Hence, the practices to grow geoduck larvae in a flow-through system also need to be resolved for places which have constrains in tank volume or space.

In addition to optimizing culture conditions, any effort on optimizing *P*. *zelandica* larval rearing techniques is complemented by the characterization of the larval development, which is still lacking for geoducks in terms of functional morphology. Descriptions of functional morphology are extremely useful for hatchery technicians to monitor larval development and health as well as to control finely the feeding regime during the larval rearing period.

Gribben and Hay (2003) described some characteristics of *P*. *zelandica* larvae that were reared in the lab. Zygotes developed into trochophore within 12 h and hatched to D-larvae after 24 h at 17°C. D-larvae became early umbo larvae by day 6 post fertilization, and umbo larvae by day 10. Metamorphosis began around day 16 post fertilization and most of the larvae had settled by day 18.

The growth rate of larvae was about 10 μm day^{-1}. Scanning electron microscopy (SEM) of the internal shell of straight hinge larvae indicated an absence of teeth on the provinculum at the veliger stage. Until the spat stage teeth have developed on the hinge. The development of lateral grooves at either end of the provinculum could also be seen in late umbo stage and newly metamorphosed geoducks. The shell of veligers was characterized by the prodissoconch I and II while the shell of spat were distinguished by the dissoconch. These morphological descriptions are important for larval identification. However, functional interpretation over different developmental stages, which is critical for larval rearing, is still missing.

Settlement and Metamorphosis

Major bottlenecks in hatchery production are that larval settlement rate, metamorphosis rate and spat survival are low and the spat production is inefficient (i.e. uncontrolled and very space consuming). Metamorphosis of bivalve larvae is known to be induced by physical, biological and chemical cues. In the wild, *P. generosa* beds were associated with chaetopterid polychaete mats (Cooper and Pease, 1988). The dialysate of sand from a geoduck bed increased the metamorphosis rate (King, 1986). However, the cues that induce settlement in geoduck larvae are still unknown. Some chemical cues such as potassium ion (K^+) and γ-aminobutyric acid (GABA), and acetylcholine (ACh) have been proven to induce settlement of mussels (Young et al., 2011; Alfaro et al., 2011), clams (García-Lavandeira et al., 2005) and oysters (Doroudi and Southgate, 2002; García-Lavandeira et al., 2005). As a starting point, information is needed with regards to critical stages in geoduck larval development, such as metamorphosis, and how these processes can be synchronized to achieve effective and efficient spat production. No study has

examined those aspects for geoducks so far. Hence, in practice geoduck larvae are simply allowed to metamorphose with no special treatment (Feldman et al., 2004).

Nursery culture of spat

The early spat stage is still a “black box” phase for geoduck hatchery production (N. King pers. comm.). At metamorphosis, the swimming function of the velum is lost; spines develop on the growing edge of the shell of spat, which then begin to crawl actively, followed by burrowing using their foot (Goodwin et al., 1979; Goodwin and Pease, 1989). This spat stage lasts for 2–4 weeks (Goodwin and Pease, 1989) and the post-larvae pedal-palp feed using their foot to transfer detritus to the mouth (King, 1986). Also, *P*. *generosa* spat produced byssal threads to attach to substratum surfaces or form long threads that act as ‘parachutes’ to help disperse them on ocean currents (Goodwin and Pease, 1989).

Feldman et al. (2004) described the spat nursing in Taylor Shellfish hatchery (Washington, USA). The competent *P*. *generosa* larvae (about 0.3 - 0.4 mm shell length) were placed into primary nursery systems that consisted of upwellers or downwellers and were supplied with 1 µm filtered seawater at 15 - 17°C and cultured microalgae. Pediveliger geoducks were placed into individual screen silos at a stocking density of approximately 150,000 pediveligers per 20“ diameter silo, or 450,000 per upweller/downweller. Silos were placed on down welling mode and maintained for several days until pediveligers have become byssal plantigrades and attach to the screens. At this point, spat may be handled and removed with a gentle flow of filtered seawater and placed into secondary nursery systems. These consist of large screens filled with clean, washed sand supplied with under-substrate plumbing

designed to generate a slight downwelling effect. The byssal plantigrades were supplied with filtered seawater and clean microalgae until large enough for tertiary field nursery systems (Feldman et al., 2014).

The main difficulty in developing culture techniques for geoduck spat has been the requirement of sand to allow burrowing. At the size of 1.5-2.0 mm shell length, *P*. *generosa* spat became juveniles, which take on the general morphology of adults and tend to favour burrowing activity over crawling on the substratum surface (King, 1986). However, within 0.3 to 2 mm shell length stage, the sand requirements have not been identified for geoducks. With geoduck as well as other burrowing clam species, when spat are transferred into sand, it is very hard to know what happens during the following time and are until they are washed out of the sand.

Nursery culture of early juvenile

In Taylor Shellfish hatchery and Nova Harvested Ltd., when geoducks become juvenile plantigrade they are transferred to tertiary field nursery systems. Tertiary systems consist either of large outdoor tanks or raceways or totes supplied with a similar sand substrate and flowing seawater, or field based "kiddiepools" supplied with clean sand and a mesh cover to eliminate predators that are placed onto the low intertidal beach (Feldman et al., 2004; Dodd, 2014).

Temperature is a very important consideration for successful nursery production of early juvenile. Recently, Arney et al. (2015) examined the effects of temperature and ration on the growth and survival of *P. generosa* juveniles. The growth rate of *P. generosa* juveniles increased with temperature from 7 to 19°C. However, there was no difference in ash free dry weight (AFDW) between 7°C and 19°C, nor between 11°C and 15°C. The latter set had significantly

higher AFDW than the former set. Also, they recommended increasing rations from 4×10^6 to 64×10^6 equivalent *T. lutea* cells juvenile^{-1} day^{-1} for increasing geoduck size from 2 to 5 mm shell length.

1.2.2 Current status of geoduck field grow-out

P. *generosa* aquaculture is developed on inter-tidal lands in Puget Sound and on sub-tidal areas in British Columbia. Most of the development of aquaculture techniques has been focused on how to plant geoduck into the substrate, and how to avoid predation. Once spat become juvenile with the minimum 10 mm size, they can be transferred to grow-out beds, which can be either low-intertidal or sub-tidal beds. In inter-tidal culture systems, such as those common in Puget Sound, the geoduck juvenile or “seed” are planted directly into PVC tubes full of sand in the spring. The tubes are pushed into the sand substrate and covered with a protective mesh. The mesh is removed after the first year. The tube is left for another year and then it too is removed and re-used for the next lot of seed geoduck. Seeding density is site specific, but if the site is sufficiently productive, it can be up to one tube every square foot (Feldman et al., 2004).

In sub-tidal culture, the development of grow-out technology has been a significant challenge. An underwater self-propelled seeder has been developed to transfer the 25-30 mm sized geoduck from a hopper through seeding tines into the substrate at the desired depth. Planting at depth gives them some advantage, but the small juveniles still require further protection and are covered with a protective canopy. Without the canopy, juvenile survival is less than 10%, but with the canopy in place survival rates can be increased to 80 or 90%. Further losses will occur in the second and third year which may reduce overall survival to 50% (Feldman et al., 2004).

Intertidal surface water temperatures must also be closely monitored during planting-out of geoduck juveniles. High temperatures can kill or immobilize juveniles before they are able to bury into the substrate. Planting geoducks when beach sediments and seawater temperatures were in excess of 22°C was not recommended (Davis, 2004). Indeed, losses of juveniles because of heat stress are the major risk factors during the first year (Davis, 2004).

Besides temperature, oxygen availability also plays an important role which affects animal performance. Environmental oxygen concentration has a direct effect on the physiology of bivalves (Bayne 1971; Brand and Roberts 1973). In estuaries and intertidal locations, oxygen levels can be limiting, and in anoxic intertidal zones, aerial respiration may occur during exposure (Boyden, 1972). There is no unified consensus on critical threshold of dissolved oxygen (DO) needed for marine animal health, because these are usually species-specific but hypoxia is often defined at levels below 2 mg L^{-1}. The DO levels below 2 mg L^{-1} were indeed observed in northern Hood Canal where geoduck abundance was relatively high (Sizemore et al., 2007).

P. zelandica is a subtidal species. However, there is a desire by the nascent geoduck aquaculture industry to grow them in intertidal areas, in order to reduce significantly operational costs. Although growth and mortality are the two most important factors for aquaculturists, it is not practically feasible to directly measure these metrics in the short term, and they will fluctuate due to the integration of seasonal factors. Growth may be especially difficult to measure in the case of such a slow-growing species as geoduck, in which it takes average 6-7 years to grow to the marketable size (0.7 – 1 kg). Fortunately, physiological states, such as oxygen consumption rate and clearance rate are able to be monitored, and the aerobic scope which represents the surplus energy for

activity, reflecting the ability of organisms to fuel locomotion, feeding, growth, and reproduction (Fry, 1947) can be determined in the short-term. Thus, those physiological metrics can be good indices for animal performance ultimately manifested in growth and survival (Bernard, 1983).

Although geoducks are known to be extremely long-lived (Orensanz et al. 2004), previous studies have demonstrated that *P. zelandica* may reach reproductive maturity within just 3 years (Gribben and Heasman, 2015). While juvenile geoducks may only spend energy on building up somatic tissues, adults invest energy in both somatic and reproductive growth. It has been shown that reproduction may have a great effect on aerobic scope (Brokordt et al. 2000). It is likely that intertidally cultivated geoducks may have even longer production cycles if their growth is reduced under daily hypoxic stress or thermal stress. To inform husbandry practices, and maximize the growth and production potential of New Zealand geoducks, detailed information is needed about the effects of hypoxia and temperature on the growth and fitness of these clams.

1.3 Present Study

To pave the way for the establishment of a geoduck aquaculture industry in New Zealand, the Cawthron Institute and the Auckland University of Technology have collaborated to tackle the bottlenecks of the hatchery, nursery, and grow-out technologies. This current study is a part of a bigger program for developing geoduck aquaculture in New Zealand.

Hatchery production clearly depends on a consistent source of broodstock. However, sourcing of broodstock is problematic for New Zealand geoducks. *P. zelandica* only spawn once a year (Gribben et al., 2004) and females may produce few eggs, as is the case with *P. generosa*. Besides, the spawning time

of wild geoducks might not be suitable for production time in the existing hatchery, which is busy with other species. Hatchery production generally consists of broodstock conditioning, spawning and fertilization, embryo incubation, larval rearing, settlement and metamorphosis, spat nursing, and early juvenile nursing (Helm et al., 2004). Broodstock conditioning is to stimulate maturation with high quantity and quality of gametes (Utting and Millican, 1997) and to manipulate the gonadal cycle and spawning period of the broodstock (Castagna, 2001). Stimulation of gonad development may require the control of abiotic factors, such as temperature or photoperiod, and nutrition (quantity and quality of food) to ensure that the energy balance is positive and essential nutrients are delivered. Spawning is carried out to collect gametes. This process may involve the mature determination and spawning induction (e.g. by chemical, temperature, desiccation, and food) or stripping. Methods of collecting gametes affect the viability of gametes. Fertilization is to fuse eggs with sperms. This step may contain storing and mixing gametes so that polyspermy is avoided without compromising the number of fertilized eggs/zygotes. Embryo incubation is to incubate zygotes into embryo then veliger larvae. This stage requires stable conditions (e.g. temperature and pH), which support the normal veliger larvae hatched (e.g. full velum and shell). Larval rearing is to grow planktonic larvae until settlement/metamorphosis. This process is the most laborious, involving the monitoring of stocking density, algal ration, sanitation, as well as larval growth, survival, health, and development. Settlement and metamorphosis are a transition stage in which swimming larvae become benthic spat, which live on substrates. Factors that trigger metamorphosis might include physical (e.g. type of substrate), chemical (e.g. neuroactive compounds), and biological (e.g. bacterial biofilm) cues. Spat and

early juvenile nursing are to grow benthic spat to become juvenile. This nursing phase may require the substrate and down/upwelling system preparation and the monitoring of stocking density, algal ration, growth and health of animals. Since larvae, spat and early juvenile geoducks are extremely sensitive to adverse water quality (e.g. temperature, bacteria, and poor food quality), they quickly succumb unless close attention is paid to all aspects of larval, spat, juvenile, and algal production systems. The grow-out phase is to plant juveniles in farm systems and grow them to the marketable size. This phase may include system (e.g. seabed and suspended) and site selection (e.g. temperature, intertidal or subtidal zone) so that the growth and survival of geoducks can be maximized at different stages (juvenile and adult).

Without knowing the effects of these factors on growth and development of geoducks at different stages within their life cycle, it is difficult to establish a long-term sustainable geoduck aquaculture industry. Hence, the objectives of the present study are to investigate:

i) the maturation status of broodstock geoducks conditioned at different temperature and feeding regimes;
ii) the nutritional status of broodstock geoducks conditioned at different temperature and feeding regimes;
iii) the optimal sperm:egg ratios and embryo development of geoducks;
iv) the growth and development of larval geoducks reared at different stocking densities and algal rations using a flow-through system;
v) the effects of neuroactive compounds on metamorphosis of larval geoducks;
vi) the size effects on geoduck physiology;

vii) the thermal effects on physiological rates of juvenile and adult geoducks; and

viii) the hypoxic effects on physiological rates of juvenile and adult geoducks.

1.4 Thesis Structure

The overall objectives of this dissertation were to identify conditions and practices to improve the broodstock conditioning, fertilization, larval rearing, spat nursing, and juvenile growing of *P. zelandica*. To achieve these objectives, 13 experiments were conducted throughout the life cycle of *P. zelandica*. Chapters 2 to 9 were written as original research papers. They are formatted differently depending on the journal's format. The general introduction is where the study context is laid out (Chapter 1). The general discussion brings together all the information in the previous chapters to discuss the potential for geoduck aquaculture in New Zealand.

With respect to broodstock conditioning, young adult geoducks were subjected to various levels of temperature and food rations (Chapter 2 and 3). Their maturation was examined by histological indices (e.g. gonadosomatic, connective tissue occupation, matured ratio; Chapter 2). The practical protocol for broodstock conditioning was discussed in this chapter. Besides, the nutrient status of conditioned broodstock, geoducks were examined by biological indices (e.g. glycogen, protein, lipid, and fatty acids; Chapter 3). The importance of positive energy balance and some essential fatty acids was discussed. In regard to spawning and fertilization, different sperm:egg ratios were tested to avoid polyspermy, but maintain the highest normal embryo ratio (Chapter 4). The embryonic development was described based on photos taken by scanning electron microscopy. In addition, a practical protocol for spawning and

fertilization was discussed in this chapter. In light of larval rearing, different practices, and results of survival and growth from different batches were reported (Chapter 5). Also, the larval development in terms of functional morphology was described. Identical practices related to feeding level and stocking density were determined as the foundation for future research. In the search for effective induction of metamorphosis, different neuroactive compounds (potassium ions, acetylcholine, and epinephrine) were tested to induce metamorphosis (Chapter 6). The effects of compounds on metamorphosis and other potential inducers were discussed in this chapter. As for the influences of size on morphology and physiology, the allometric coefficients of weight-length relationship and of physiological rates - size (e.g. respiration rate and clearance rate) were determined (Chapter 7). The influence of orientation on the energetic acquisition and expenditure of large geoducks was also examined. In the matter of thermal windows for grow-out, aerobic scope and clearance rate were evaluated at five levels of temperature for two size classes (Chapter 8). The suitable temperatures for growing juvenile and young adult geoducks were discussed in this chapter. On the subject of site selection (intertidal vs. subtidal), aerobic scopes and oxygen regulation capacity were evaluated in progressive hypoxia for two size classes (Chapter 9). The suitable sites were discussed for growing juvenile and young adult geoducks.

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CHAPTER 2 - Broodstock conditioning of New Zealand geoduck (*Panopea zelandica*) within different temperature and feeding ration regimes

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Abstract

Two-year old New Zealand geoducks (*Panopea zelandica*, Quoy and Gaimard, 1835) were conditioned within combinations of three water temperatures (7–8, 11–12, and 16–17°C) and three feeding rations (10,000, 50,000, and 100,000 cells mL^{-1} of *Chaetoceros muelleri* and *Tisochrysis lutea*, 1:1 cell counts) for 73 days. Similar percent matured and dry condition index values were observed among temperatures. However, significantly higher dry gonadosomatic indices (GSI_{dw}) were recorded at 8 and 12°C. Although no difference was detected in percentage of spawned individuals and connective tissue occupation indices, a higher percent matured were recorded when fed 10,000 and 50,000 cells mL^{-1}. A reference group conditioned in pond water became matured two months later than the other 9 experimental groups, but GSI_{dw} were similar. To maximize reproductive output, we suggest that 2-year old *P. zelandica* may be conditioned in pond water for a month and then in 8 or 12°C seawater with 50,000 cells mL^{-1}.

2.1 Introduction

The New Zealand geoduck clam, *Panopea zelandica*, has been identified as a new potential species to add to the country's aquaculture exports, which are aimed to reach 1 billion dollars by 2025 (Carter 2012). While this species has an excellent potential to achieve a high commercial value, there are many obstacles that need to be overcome before reliable production and markets can be established. Unlike other bivalve species, such as Pacific oyster (*Crassostrea gigas*) and Greenshell™ mussel (*Perna canaliculus*), *P. zelandica* spat cannot be obtained commercially from the wild. Thus, a reliable geoduck hatchery production must be established to supply on-growing farmers. In addition, hatchery production depends on a consistent source of broodstock, which is problematic for geoduck since this species has a long maturation period with one spawning peak per year (Gribben et al. 2004). An added problem is that *P. zelandica* females may produce few eggs (about 2 million eggs per female; personal observations), as is the case with *P. generosa* females, which produce about 2-10 million eggs (Beattie 1994). With these bottlenecks to overcome, it is clear that early research efforts to commercialize this species need to be focused on broodstock conditioning and larval production. Specifically, temperature and feeding ration are well-known to affect broodstock conditioning of shellfish.

It is well-know that temperature has an important influence on the reproductive development of bivalves (Bayne 1976; Utting & Millican 1997; Helm et al. 2004). For geoducks, reproductive cycles have been shown to have a strong association with season for the Pacific species, *P. generosa* (Andersen 1971; Goodwin 1976; Sloan & Robinson 1984; Campbell & Ming 2003), the Southern species, *P. abbreviata* (van der Molen et al. 2007) and the Cortes

species, *P. globosa* (Calderón-Aguilera et al. 2010b). This seasonal reproductive behaviour suggests a strong influence of water temperature, although the particular patterns are species specific. For example, the gametogenic development of *P. generosa* starts in late autumn and winter when water temperatures are low (8°C), and spawning occurs in spring/summer when water temperatures rise to 11-12°C (Sloan & Robinson 1984). However, gametogenesis of *P. globosa* begins in mid-autumn,when the water temperature is warmer (over 26°C), and gametes are released during cooler periods (16°C) (Calderón-Aguilera et al. 2010b). Conversely, ripe *P. abrreviata* females are found throughout the year, and higher proportions of spawning individuals are encountered during the colder months (van der Molen et al. 2007). Recently, Marshall et al. (2012) found that *P. generosa* held at 7 and 11°C displayed a more advanced state of reproductive development than individuals that were maintained at 15 and 19°C. For *P. zelandica*, water temperature has been suggested to modulate the time to maturity as well as spawning (Gribben et al. 2004). In the only study to investigate wild *P. zelandica*, Gribben et al. (2004) suggested that gamete production was three months longer at cooler sites (Shelly Bay; 11-15°C) compared with warmer sites (Kennedy Bay; 15-19°C) in northern New Zealand (Gribben et al. 2004). These studies suggest that temperature may have an important effect on reproductive conditioning of *P. zelandica* for aquaculture purposes. However, specific studies to identify the effect of temperature on maturity and spawning of *P. zelandica* has been absent until now. Gribben & Hay (2003) attempted to spawn wild broodstock from Shelly Bay and Kennedy Bay in the laboratory, but those attempts failed without chemical induction. Therefore, establishing the appropriate temperature for gonadal growth and development in *P. zelandica*

was considered to be the first critical step to establish successful reproductive conditioning protocols for this species.

In addition to temperature, food quality and quantity have been shown to be deterministic factors in bivalve conditioning, since gonad development and maturation depend on sufficient and essential nutrient requirements (Utting & Spencer 1991). Conditioning experiments with *P. generosa* have shown that rations of 4.0×10^9 and 5.6×10^9 cells^{-1} geoduck^{-1} day^{-1} of a mixture of *Chaetoceros muelleri* and *Tisochrysis lutea* (formerly known as *Isochrysis affinis galbana* or *T-ISO clone*; Bendif et al. 2013) achieved the highest survival and maturation rates for this species (Marshall 2012). In addition, Marshall (2012) tested the efficiency of three different microalgal species (*Tisochrysis sp*., *C. muelleri*, and *Dunaliella tertiolecta*), and one mixed diet (*Tisochrysis sp*. plus *C. muelleri*), on reproductive development of *P. generosa*, but was unable to detect any differences based on the quality of these feeds. Furthermore, García-Esquivel et al. (2013) found that *P. globosa* could be matured successfully with *T. lutea* as the sole diet at a ration of 16.4×10^9 cells^{-1} geoduck^{-1} day^{-1}. However, optimal broodstock feeding requirements are strongly species-specific, and this information is lacking for *P. zelandica*. For practical purposes, broodstock conditioning of bivalve species has tended to use microalgal diets that are both readily available and have shown some success for other species. One such diet is a combination of *Chaetoceros sp*. (rich in DHA) and *Tisochrysis sp*. (rich in EPA), which has been used successfully with bivalves such as the Pacific geoduck *P. generosa* (Marshall 2012) and the Pacific oyster *C. gigas* (Helm et al. 2004). However, appropriate rations may vary greatly from species to species, since utilisation of glycogen

reserves for lipid synthesis during vitellogenes is differ from species to species (Gabbott 1983).

External factors, mainly, temperature and feeding ration have been demonstrated to play an important role on the scope for growth of marine bivalves (Bayne & Newell 1983; MacDonald & Thompson 1986; Bayne & Hawkins 1990; Thompson et al. 1996). Ultimately, identifying the correct ration in relation to a specific temperature (i.e. scope for growth) is critical. However, no study combining the effects of temperature and food ration on reproduction has been carried out for geoducks. Thus, the aim of this study is to investigate the effects of water temperature and food ration on the conditioning of *P. zelandica* broodstock for aquaculture purposes.

2.2 Methods and Materials

2.2.1 Source of broodstock

Two-year old geoducks, which were grown from gametes at the Cawthron Institute, Nelson, New Zealand, were utilised as broodstock. The animals used were produced from the same set of parents, and were reared under the same culturing conditions. This common history provided a reduced variability in reproductive condition due to season, location, age and genetic makeup.

The geoducks were dug up from the nursery tank (0.8 m height x 1 m diameter with a 0.5 m sand layer), and immediately cleaned of biofoulings (mostly ascidians). One hundred individuals of similar shell lengths (the anterior-posterior axis of the right valve) and wet weights were selected. Mean (±SD) shell lengths were 57.8±5.5 mm and mean (±SD) wet weights were 44.8±9.4 g. These were relatively small individuals since it was expected that this information could be used to speed up the breeding process in the future.

2.2.2 Production of microalgae

Monocultures of two microalgal strains that are typically grown at the Cawthron Institute, Nelson, were selected for this experiment: *Tisochrysis lutea* and *Chaetoceros muelleri*. The microalgae were cultivated according to standard hatchery practices in 40-L plastic bags (Kaspar et al. 2014). The cultures were axenic and continuous with Conway media. The culture conditions included: temperature of 20–23°C; continuous light at 750–850 lx from cold white fluorescent tubes; salinity of 35 ppt pasteurized water at 95°C; and aeration of 2.4 L min^{-1} with CO_2 addition at 0.2% v:v. For *C. muelleri*, additional silicates (30 mL of a 30-g L^{-1} stock solution) were added directly to the 40-L bags every second day.

2.2.3 Conditioning experiment

Geoducks were step-wise acclimatized at 8, 12 and 16°C for three weeks. During the acclimation phase the geoducks were continuously fed at a ration of 35,000–40,000 cells mL^{-1} (*C. muelleri* : *T. lutea* = 1:1 by cell count). The broodstock conditioning experiment involved a completely randomized factorial design, with three water temperatures (7–8, 11–12, and 16–17°C) and three feeding rations (10,000, 50,000, and 100,000 cells mL^{-1} of a 1:1 cell count mixture of *C. muelleri* and *T. lutea*) (Fig. 2.1). These temperature levels were selected based on the temperature range for wild populations and feeding rations were selected according to previous clearance rates experiments for two-year old *P. zelandica* (Le et al. unpublished data). Salinity was 35 ppt throughout the experiment. Each of the nine treatments included three replicate tanks, each with 3 geoducks inside. A separate reference group of nine geoducks were exposed to pond water to generally compare the experimental animals to those grown in more “natural” conditions. However, the reference

group was not included in the statistical analyses. The condition period lasted for 73 days. The experimental set up included a flow-through system in which water and microalgae flowed at a rate of 160 mL min^{-1} from header tanks to the experimental tanks (10 L PVC tanks). Each of the 27 tanks contained three geoducks, placed vertically inside individual 400 mL-plastic bottles containing 300 g of sand from the nursery tank (grain size between 1–2.4 mm in diameter). These sand bottles mimicked the natural substrate conditions, and allowed for minimal handling disturbance to the animals.

2.2.4 Sampling

Five geoducks were sacrificed at day 0 for histological gonad analysis, and wet weight and dry weight measurements. At day 36 and 73, another 3 randomly selected geoducks per treatment, respectively, were sacrificed for histological and morphometric analyses. The remaining geoducks were induced to spawn by thermal shock on day 73 following procedures in Helm et al. (2004). The percent spawning individuals was calculated for each treatment.

2.2.5 Histological analysis

A section containing gonad tissue was cut from the right side of the posterior end of the visceral mass of each geoduck (Marshall et al. 2012). The histological samples were prepared following Howard & Smith (1983). Briefly, the gonads were fixed in a formalin solution for 48 h and then transferred to 70% ethanol for storage. The samples were cut into 4–5 sub-sections, dehydrated using a series of ascending ethanol, and eventually embedded in paraffin wax. Embedded gonad samples were sectioned to 5 µm, stained with hematoxylin-eosin, and mounted on slides for examination under a microscope (Olympus BX41).

Sex was determined by the presence of oocytes or spermatocytes. The gonad stages were classified as early active, late active, ripe, partially spent, or spent/resorbed according to the most dominant stage of at least ten randomly chosen follicles in each slide (Gribben et al. 2004). The stages were then grouped into two categories: mature (late active, ripe, partially spent) or immature (spent/resorbed, early active) (Marshall et al. 2012).

2.2.6 Connective tissue occupation index

Connective tissue occupation index (COI) is defined as the ratio of connective tissue surface area to total gonadal surface area (including connective tissue, follicles, and vacuoles, but excluding digestive gland), was used as an indicator of gonad degeneration. This index allowed for the comparison of COI among treatments regardless of sex (Marshall et al. 2012). The region of connective tissue was determined colorimetrically using image processing software, ImageJ (http://rsb.info.nih.gov/ij/index.html). Images were filtered by convolve and Gaussian blur. Subsequently their thresholds were adjusted in 8-bit greyscale (0 = black, 255 = white). Within that color scale, connective tissue and the follicle were distinguished. The COI was the average of the area fraction of 40 fields (each field 48070 μm^2) from each gonad histological sample. This method was adapted from Marshall et al. (2012).

2.2.7 Condition index

Condition index (CI) which indicated the meat condition was calculated as:

$$CI = (T \times 100\%) / S \qquad \text{(Walne 1976)}$$

where, CI is the condition index (%), T is the total soft-tissue weight (g), and S is the shell weight (g). This index was calculated for wet (CI_{ww}) and dry (CI_{dw}) weights. There was no increase in shell length during the experimental period; hence, changes in shell weight were assumed negligible based on the

allometric relationship models of age to shell weight in Sloan & Robinson (1984) and Bureau et al. (2003).

2.2.8 Gonadosomatic index

The gonadosomatic index (GSI) which correlated with reproductive cycle of wild *P. generosa* (Sloan & Robinson 1984) and *P. zelandica* (Gribben et al. 2004), was calculated as:

$$GSI = (V \times 100\%) / T \quad \text{(Sloan \& Robinson 1984)}$$

where, GSI is the gonadosomatic index (%), V is the visceral mass weight (g), and T is the total soft-tissue weight without valves (g). This index was calculated for wet (GSI_{ww}) and dry (GSI_{dw}) tissues. Because the gonad and visceral mass were not separated, the entire visceral weight was used in the numerator of the GSI instead of goand weight (Gribben et al. 2004).

2.2.9 Statistical analyses

Due to the low number of animals available for this experiment, the number of observation units per replicate was low. Thus, non-parametric two-way ANOVA tests based on permutations (unrestricted with 999 random permutations and a seed integer of 5) were used to test the interaction effect and main effects of temperature and feeding ration on condition index, gonadosomatic index, connective tissue occupation index, maturation and spawn for each sampling event. If the interaction effect or the main effects were significant, a pair-wise comparison among treatments was analysed with a *t*-test. The condition index and gonadosomatic index data did not need transformation to satisfy statistical requirements, but the connective tissue occupation index data were arcsine transformed. The maturation and spawning data were converted to presence/absence. Non-parametric two-way ANOVAs were conducted on Bray–Curtis distances calculated from raw and transformed data using the

FORTRAN program PERMANOVA (Anderson, 2001). By using permutations, the tests did not need specific assumptions concerning the number of variables or the nature of their individual distributions or correlations.

2.3 Results

Results from the conditioning experiment indicate that there was no significant difference among temperature or feeding ration treatments for geoduck wet (CI_{ww}) and dry (CI_{dw}) weights after the first 36 days of the experiment (Fig. 2.2; Table 2.1). However, after 73 days, there was a general trend of increasing geoduck CI_{ww} and CI_{dw} with increasing feeding ration for the medium (11–12°C) and high (16–17°C) temperature treatments. A different pattern was observed at low temperatures (7–8°C), where a medium feeding ration (50,000 cells mL^{-1}) resulted in the highest geoduck CI_{ww} and CI_{dw} values (Mean±SD) of 416.4±28.6 and 109.1±8.9%, respectively. These values were similar to those of geoducks conditioned in pond water (reference group), which were 391.7±2.5 and 113.2±12.1% for CI_{ww} and CI_{dw}, respectively. These values were not as high (although not significantly different) as those of individuals sampled at the start of the experiment (day 0), which were 461.0±54.7% and 122.4±10.6%, respectively for CI_{ww} and CI_{dw}. Two-way ANOVAs on Bray-Curtis distances analyses resulted in significant temperature and food ration treatments and interaction for CI_{ww} and a significant food ration treatment and interaction for CI_{dw} (Table 2.1). These results reflect the different trends of feeding ration effects among temperatures.

Geoduck gonadosomatic analyses resulted in decreasing GSI_{ww} and GSI_{dw} values with increasing temperature after the first 36 days (Fig. 2.3; Table 2.1). The highest values (Mean±SD) of 29.8±5.4 and 32.6±6.0% for GSI_{ww} and GSI_{dw}, respectively, were observed in geoducks conditioned at low

temperatures. These values were similar to those of geoducks in the reference group, which were 30.9±1.8 and 30.6±5.2% for GSI_{ww} and GSI_{dw}, respectively. However, after 73 days, the highest GSI_{ww} and GSI_{dw} values were observed in geoduck conditions at medium temperatures (25.4±3.0 and 25.8±4.1%, respectively), while those of geoducks in the reference were 22.4±0.1 and 29.4±6.5% for GSI_{ww} and GSI_{dw}, respectively. Conversely, GSI_{ww} and GSI_{dw} values for geoducks sampled on day 0 were 20.7±5.2 and 25.0±6.5%, respectively. Results from two-way ANOVAs on Bray-Curtis distances analyses indicate significant differences among temperatures and non-significant differences among food rations and interactions for both GSI_{ww} and GSI_{dw} after 36 and 73 days (Table 2.1).

The connective tissue occupation index did not differ significantly among temperature or feeding ration treatments after 36 days or 73 days (Fig. 2.4; Table 2.1). On day 36, the high temperature and high feeding ration treatment had the highest COI values (83.7±9.6%, mean±SD), while the lowest COI values were observed in the low temperature and medium feeding ration group (47.0±34.8%). After 73 days, the low temperature and medium feeding ration group still had the lowest COI values (32.0±34.9%), and the highest values were recorded in the medium temperature and high feeding ration (91.5±8.9%). The COI of the reference group on day 73 and initial group were 82.4±5.4 and 76.8±4.2%, respectively (Fig. 2.4).

The percentage of mature gonads was not significantly different among temperature and feeding ration treatments after 36 days (Fig. 2.5; Table 2.1), but the feeding ration alone was significant for the 73-day data set (Table 2.1). Geoducks fed medium feeding rations had a significantly higher percentage (77.8%) of mature gonads than those fed low and high feeding rations after the

73-day experiment (Fig. 2.5). The reference group had the lowest percentages (0%) of mature gonads compared to all feeding ration groups. The percentages of geoducks which spawned after the 73-day conditioning experiment were not significantly different among temperature and feeding ration treatments (Fig. 2.6; Table 2.1).

2.4 Discussion

To determine which temperature and feeding ration were more appropriate for geoduck broodstock conditioning, we examined the data set based on two priorities. The first priority was evidence of maturity and spawning. The second priority was the reproductive development indicators (GSIs and CIs). The gonadosomatic index has been correlated with reproductive state in wild populations of *P. generosa* (Sloan & Robinson 1984) and *P. zelandica* (Gribben et al. 2004). Condition indices are indicators of stress or sexual activity, and low values of these indices indicate that a major biological effort has been expended, either as maintenance energy under poor environmental conditions or disease, or in the production or release of gametes (Lucas & Beninger 1985). Although wet and dry gonadosomatic and condition indices were measured, we based our interpretations on GSI_{dw} and CI_{dw}, since the use of the dry indices in bivalves eliminates the bias due to water content fluctuations of whole tissue samples (Lucas & Beninger 1985; Gribben et al. 2004; Marshall et al. 2012). Results from our study indicate that although high temperatures were acceptable for conditioning geoducks, low and medium temperatures were more appropriate to condition 2-year old *P. zelandica* broodstock. Based on the percentage of mature gonads, spawned geoducks, and COI values, there was no difference in maturity among all three temperature levels. However, results from GSI_{dw} analyses indicate that geoducks had a higher gonad material ratio

when exposed to medium and low temperatures. GSI_{dw} values on day 36 and 73 at medium (25 and 25%, respectively) and low (32 and 23%, respectively) temperatures were higher than GSI_{dw} values at high (22 and 19%, respectively) temperatures. In addition, CI_{dw} values were not different among the three temperatures and the dry weight gonad:shell weight ratio of the high temperature treatment was significantly higher than that of the low and medium temperature treatments (data not shown). These results confirmed that the differences in GSI_{dw} values were due to the development of reproductive products.

There is limited information on the effect of temperature on gonad development of *P. zelandica*. However, previous studies on the reproductive cycle of wild geoducks support our result that geoducks may become mature when exposed to either high or low temperatures (Gribben et al. 2004; van der Molen et al. 2007). Gametogenic development of *P. zelandica* from Kennedy Bay started at low temperatures (11°C), while that of *P. zelandica* from Shelly Bay started at high temperatures (18°C) (Gribben et al. 2004). Also, *P. abbreviata* were found ripe for most of the year when exposed to a temperature range of 8 to 20°C (van de Molen et al. 2007). The lack of significant influence of a particular temperature window on the percentage of mature gonads in our study also has been observed in other bivalve species. Toba and Miyama (1995) concluded that temperatures between 10 and 27°C were probably not a limiting factor in the reproductive activity of *Ruditapes philippinarum*. Also, if the amount of food ingested was similar, then temperature differences of 4°C had no significant impact on the rate of gonadal development of *R. philippinarum* (Delgado & Camacho 2007). In addition, Navarro et al. (2000) rejected the effect of temperature within the range studied (16–20°C) on the gonadal

development of *Argopecten purpuratus*. Moreover, an increase in temperature within 2–15°C did not affect the speed of gametogenesis in the mussel *Mytilus edulis*, except at temperatures close to their lower LT_{50} (Gosling 2003). In fact, our temperature range was similar to that of Marshall et al. (2012), who found no difference in the percentage of mature gonads of *P. genero*sa at a range between 7 and 15°C, but found a difference when the exposure was 19°C.

For many marine species, food rather than temperature is the major factor determining the timing of gametogenesis (Gosling 2003). Our study showed that medium feeding rations were the most appropriate for conditioning 2-year old *P. zelandica*, while the low rations were also acceptable. According to the percentage of mature gonads, medium and low feeding rations (55.6 and 77.8%) were superior to high rations (11.1%). However, based on the percentage of spawned geoducks, COI, and GSI_{dw} values, there was no difference among the three feeding rations. Furthermore, CI_{dw} values indicate that geoducks exposed to medium feeding rations had more meat ratio (96.4%) than those in the low feeding rations (80.5%).

Previous studies of conditioning other geoduck species support our results that feeding ration influences gametogensis development. However, results from feeding ration values among studies are widely different and difficult to compare. To make a feasible comparison among studies, we standardized the feeding ration units in Marshall (2012) and our studies to percentage dry weight of algae/dry weight of tissue. Marshall (2012) used a range of rations: 0.8×10^9, 4.0×10^9, 5.6×10^9, 7.2×10^9, and 10×10^9 cells geoduck^{-1} day^{-1}, which were equivalent to 0.02, 0.1, 0.14, 0.18, and 0.25% dry tissue weight. Our feeding rations (10,000, 50,000, and 100,000 cells mL^{-1}) were equivalent to 0.58×10^9, 2.88×10^9, and 5.76×10^9 cells geoduck^{-1} day^{-1},

respectively. These values were equivalent to 0.33, 1.65, and 3.3% dry tissue weight, respectively. Marshall (2012) found that a 0.25% ration caused *P. generosa* mortality, while the high and low rations (0.18 and 0.02%) resulted in less gonadal material than those in the medium rations (0.14 and 0.1%). In addition, *P. globosa* could become mature when exposed to a 0.31% dry tissue weight feeding ration without compromising survival (García-Esquivel et al. 2013). On the other hand, our study showed that the most appropriate feeding ration was 1.6%, and a feeding ration of 0.33% was acceptable. We also observed the least number of mature *P. zelandica* at a feeding ration of 3.3%. Another potential difference for the result differences among studies could be the size of the animals studied. Our specimens were on average 44 g of live wet weight, while Marshall (2012) and García-Esquivel et al. (2013) used 1200–1600 g live wet weight animals. A smaller animal has proportionally higher metabolic demands than larger animals (Hamburger et al. 1983; Brougrier et al. 1995) and therefore requires proportionally more food. The rapid growth of wild geoducks during the first few years (Goodwin & Pease 1989; Gribben & Creese 2005; Calderón-Aguilera et al. 2010a; Pérez-Valencia & Aragón-Noriega 2013) supports this interpretation, since old adult geoducks require energy for only maintenance and reproductive growth, while juvenile and young adult geoducks still utilized energy for maintenance, reproductive growth, and additional somatic growth.

In comparison with other bivalve species (i.e., oysters, clams, scallops) reported by Helm et al. (2004), the appropriate feeding ration for geoduck conditioning in our study and previous studies was lower (0.1–1.6%) than theirs (2–6%). There may be two reasons for successful maturation at low feeding rations: 1) lower metabolic demands of larger animals (mentioned above) and

2) the fact that geoducks belong to a bivalve group which relies on endogeneous reserves for gamete development (Bayne 1976; Goodwin 1976). In other words, most bivalves may store reproductive energy reserves in the mantle, digestive gland (Dridi et al. 2007), and adductor muscles (Devauchelle & Mingant 1991; Ngo et al. 2006), while geoducks also store energy in their massive muscular siphon. Evidence for the use of the siphon as a reserve for gonad development also was observed in *P. generosa*, which despite being starved for three weeks, had thick gonad layers in the visceral mass (Marshall 2012). Similarly, our observations with 3-year old *P. zelandica* indicate that they became ripe after being starved for one month at 19°C and re-fed at 16°C in the following month. Some males even spawned after the first starvation month.

It also is noteworthy that the pond water group in our study had among the highest values in both CI_{dw} and GSI_{dw}. However, they did not mature until December, two months after the end of this experiment. This indicates that the pond water provided enough nutrients for geoducks to accumulate energy for gonad development, but there was a missing "trigger" to start gametogenesis earlier.

Based on the results of our study, we suggest that *P. zelandica* may be conditioned successfully with a two-phase strategy. When geoducks have yet to start gametogenesis in winter (June-August), they may be provided a medium feeding ration equivalent to 50,000 cells mL^{-1} from pond water in a flow-through system. The objective of this approach is to boost the levels of food reserves that will later be mobilized to gamete development. Following 4 to 6 weeks of a medium ration with a cold pond water temperature regime, geoducks may be transferred into hatchery conditions where temperature is

gradually increased or decreased (1 to 2°C per day) to either 8 or 12°C and a mixture of *T. lutea* and *C. muelleri* (1:1 cell count) is provided at ration 50,000 cells mL^{-1}. The objective of this conditioning approach is to trigger the gametogenesis process.

In conclusion, the temperature range provided to 2-year old geoducks did not significantly influence the gametogenic development of *P. zelandica*. Nonetheless, 8 and 12°C were preferable to maximize the development of gonad material. In contrast, feeding rations significantly influenced gonad maturation, with more matured geoducks at low and medium ration. However, a feeding ration of 50,000 cells mL^{-1} was more favorable due to the higher meat ratio. Despite having a maintained high CI, the maturation of the reference group occurred later than the experimental groups, which provides encouraging information for geoduck conditioning under laboratory conditions. Overall, this study contributes to the knowledge of conditioning young adult geoducks as a broodstock source. The implication of conditioning young adults is to shorten generation times in any future selective breeding programme. Finally, future investigations may focus on testing the effects of extreme temperatures and a wider range of feeding rations on the gametogenic process of *P. zelandica*. After a series of conditioning trials, we also recognized the limitations of having both the scarcity of broodstock for this species and the intrinsically high variability of gonad development among individuals. Thus, we have set out to develop a non-invasive method using magnetic resonance imaging (MRI) techniques to determine the sex and gonad stage of geoducks so that we can perform measurements on the same stage of individuals over time.

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Figure 2.1 Experimental design for the broodstock conditioning experiment, including three water temperatures (L = low, 7-8°C; M = medium, 11-12°C; and H = high, 16-17°C) and three feeding rations (10,000, 50,000, and 100,000 cells mL-1). Additional geoducks were sampled at the start of the experiment (Initial group) and others were conditioned in pond water as a comparative reference group.

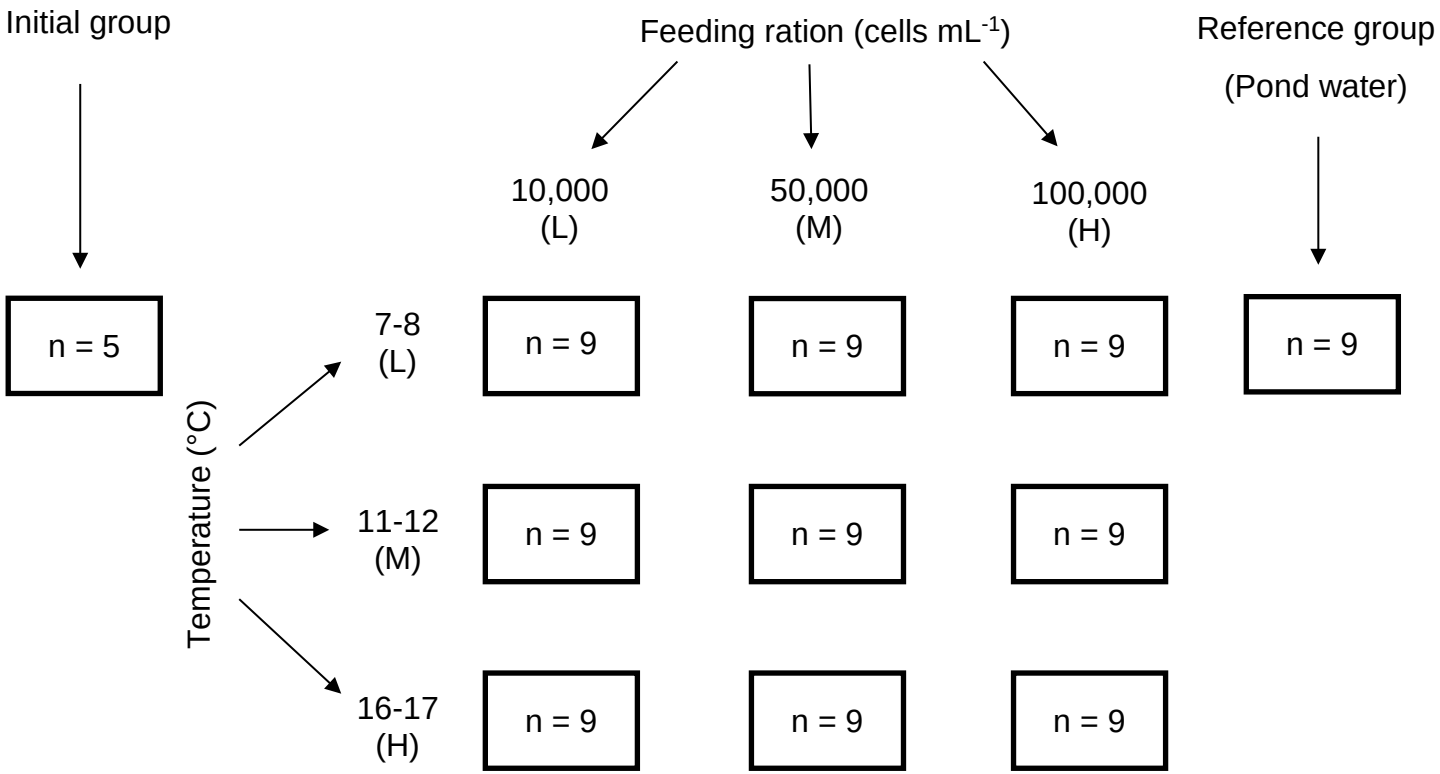
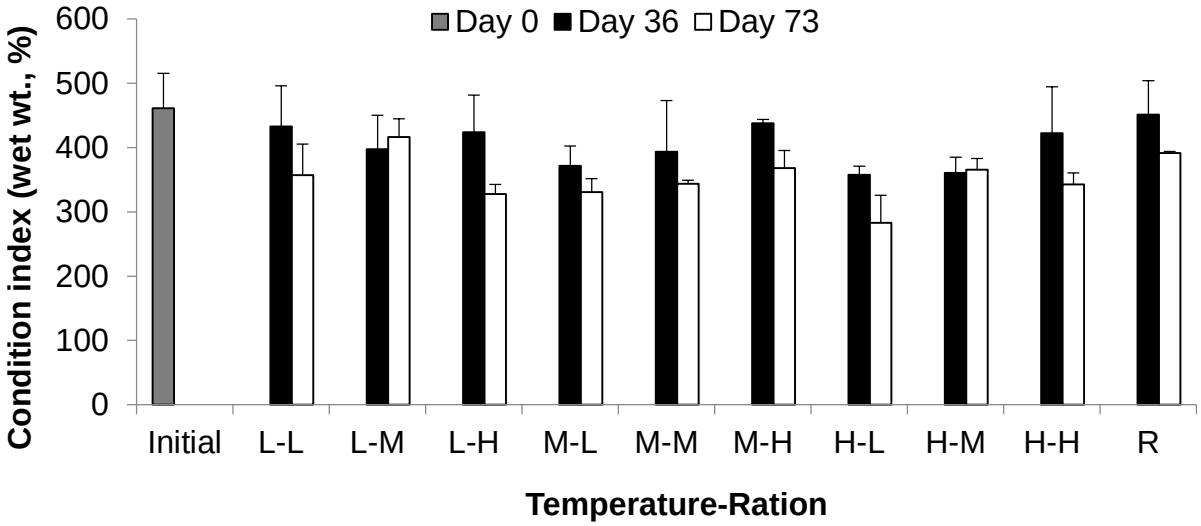


Figure 2.2 Mean condition indices (±SD) for wet and dry weight tissue of 2-year old *P. zelandica* after 36 and 73 days of conditioning in three water temperatures (L = low, 7-8°C; M = medium, 11-12°C; and H = high, 16-17°C) and three feeding rations (10,000, 50,000, and 100,000 cells mL^{-1}). Additional geoducks were sampled at the start of the experiment (initial group) and others were conditioned in pond water as a comparative reference group (R).



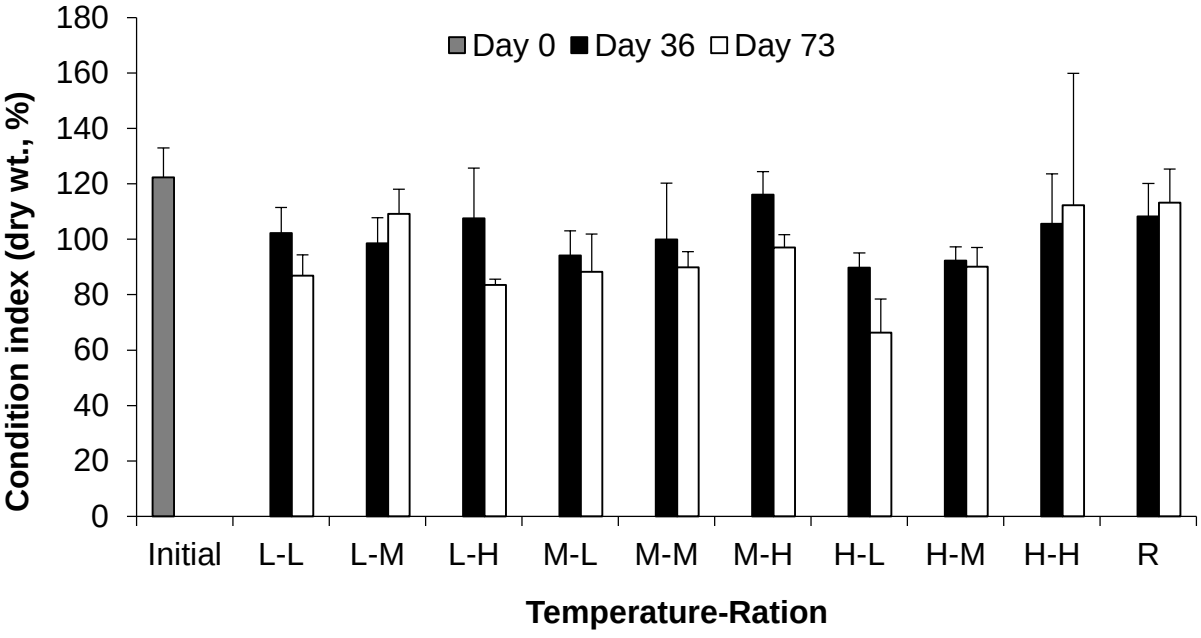
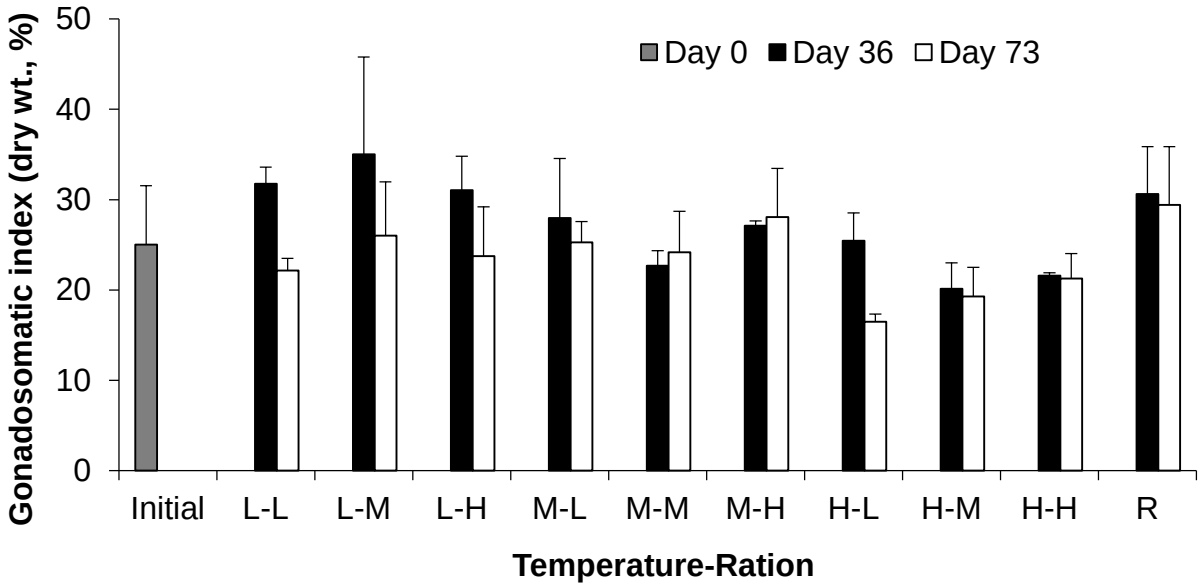


Figure 2.3 Mean gonadosomatic indices (±SD) for wet and dry weight tissues of 2-year old *P. zelandica* after 36 and 73 days of conditioning in three water temperatures (L = low, 7-8°C; M = medium, 11-12°C; and H = high, 16-17°C) and three feeding rations (10,000, 50,000, and 100,000 cells mL^{-1}). Additional geoducks were sampled at the start of the experiment (Initial group) and others were conditioned in pond water as a comparative reference group (R).



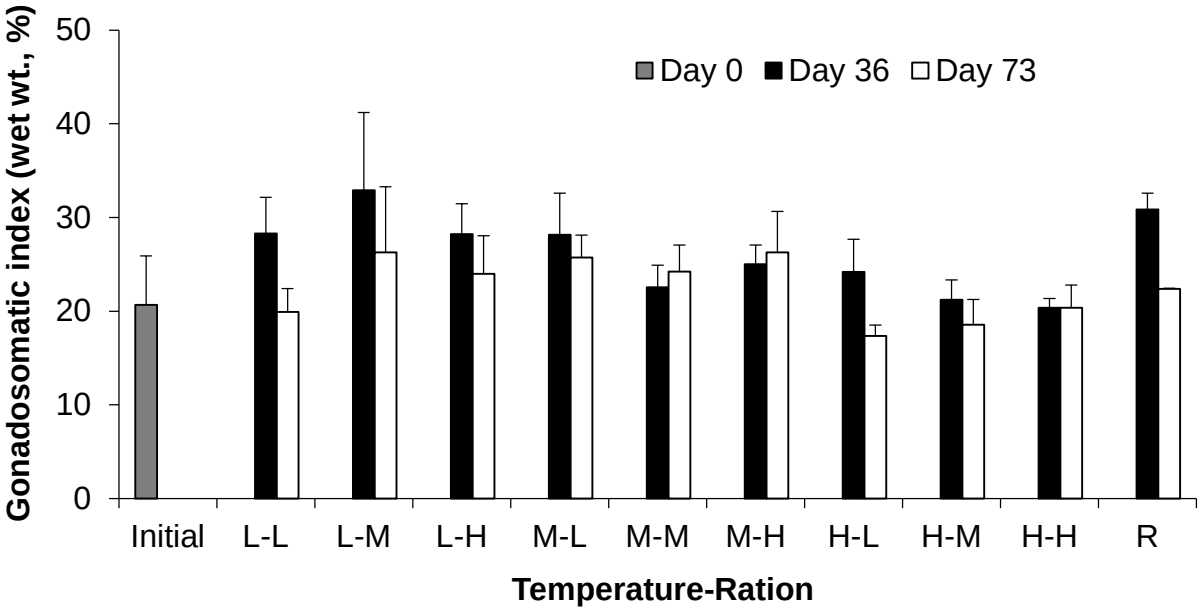


Figure 2.4 Connective tissue occupation index (±SD) of 2-year old *P. zelandica* after 36 and 73 days of conditioning in three water temperatures (L = low, 7-8°C; M = medium, 11-12°C; and H = high, 16-17°C) and three feeding rations (10,000, 50,000, and 100,000 cells mL^{-1}). Additional geoducks were sampled at the start of the experiment (Initial group) and others were conditioned in pond water as a comparative reference group (R).

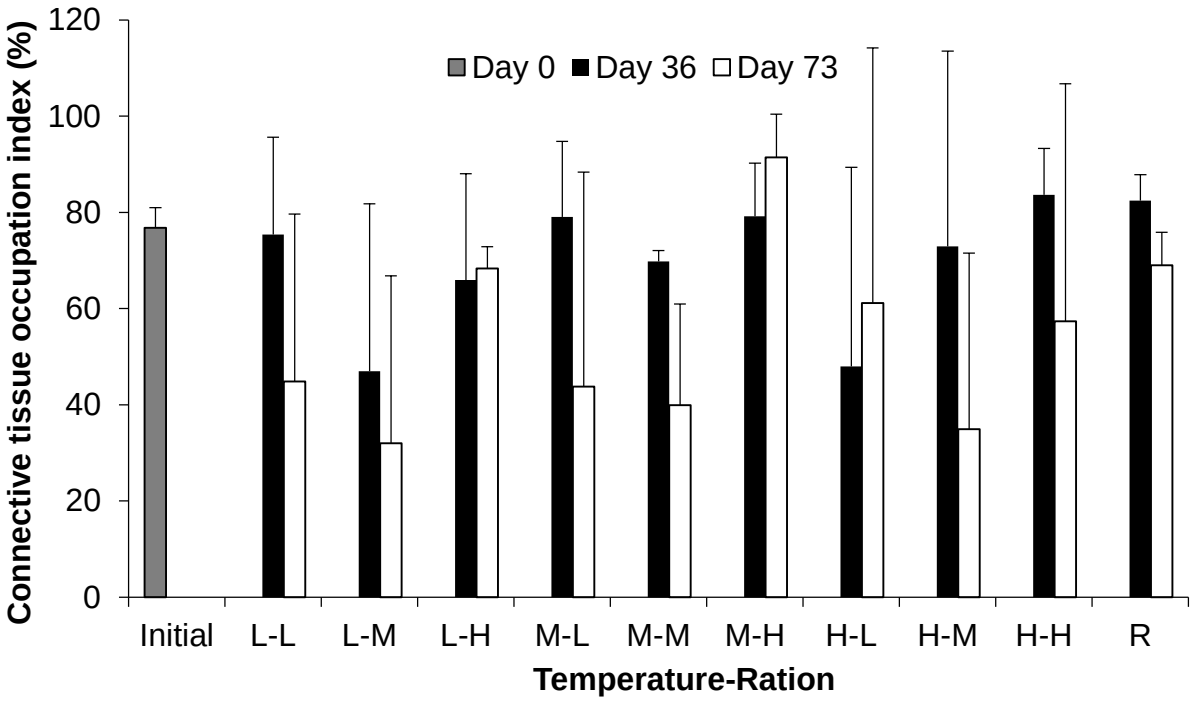


Figure 2.5 Percentage of matured 2-year old *P. zelandica* after 36 and 73 days of conditioning in three water temperatures (L = low, 7-8°C; M = medium, 11-12°C; and H = high, 16-17°C) and three feeding rations (10,000, 50,000, and 100,000 cells mL^{-1}). Additional geoducks were sampled at the start of the experiment (Initial group) and others were conditioned in pond water as a comparative reference group (R).

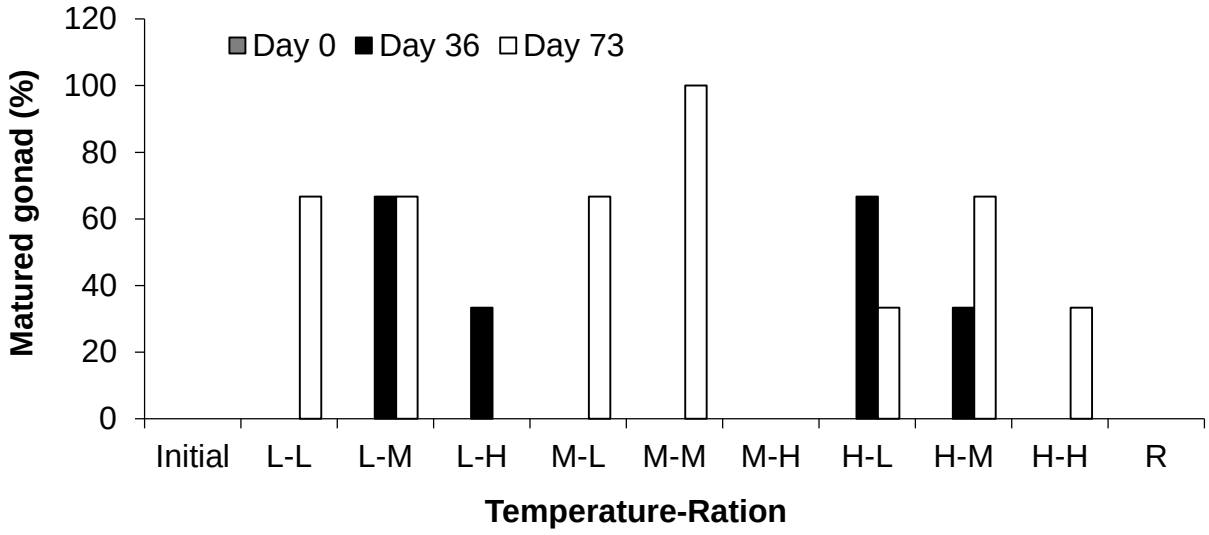


Figure 2.6 Percentage of spawned 2-year old *P. zelandica* after 73 days of conditioning in three water temperatures (L = low, 7-8°C; M = medium, 11-12°C; and H = high, 16-17°C) and three feeding rations (10,000, 50,000, and 100,000 cells mL^{-1}).

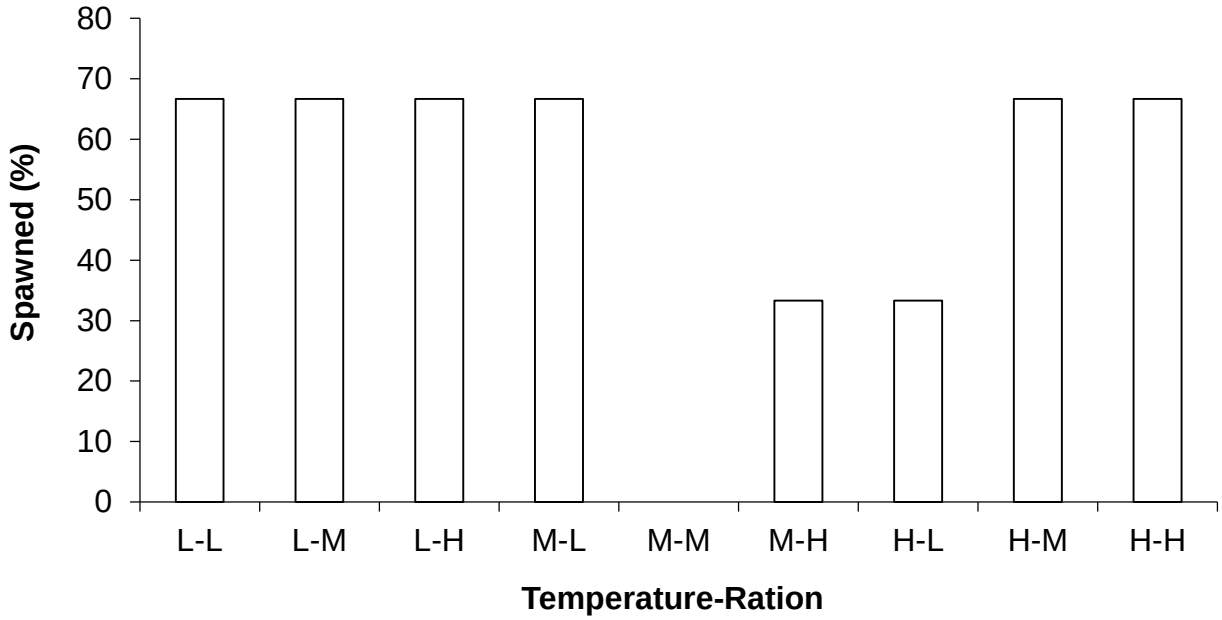


Table 2.1 Nonparametric two-way ANOVA on Bray-Curtis distances for reproductive indices of 2-year old *P. zelandica* after 36 and 73 days of conditioning in three water temperatures (L = low, 7-8°C;M = medium, 11-12°C; and H = high, 16-17°C), three feeding rations (10,000, 50,000, and 100,000 cells mL^{-1}). Bold *P*-values indicate significant tests.

					Significant pair-wise tests	
	df	MS	F	P value	Temperature	Feeding ration
Condition index (wet weight)						
Day 36						
Temperature (T)	2	49.6	1.3	0.2970		
Ration (R)	2	83.8	2.2	0.1490		
T x R	4	21.7	0.6	0.6830		
Error	18	38.8				
Day 73						
Temperature (T)	2	61.4	3.5	**0.0420**		
Ration (R)	2	128.4	7.3	**0.0040**		M with L
T x R	4	69.1	3.9	**0.0160**		
Error	18	17.6				
Condition index (dry weight)						
Day 36						
Temperature (T)	2	37.5	1.0	0.4180		
Ration (R)	2	124.9	3.2	0.0780		
T x R	4	13.6	0.3	0.8290		
Error	18	39.2				
Day 73						
Temperature (T)	2	56.4	0.9	0.4430		
Ration (R)	2	236.9	3.8	**0.0380**		M with L
T x R	4	169.6	2.7	**0.0490**		
Error	18	62.6				
Gonadosomatic index (wet weight)						
Day 36						
Temperature (T)	2	480.7	9.7	**0.0020**	H with M, L	
Ration (R)	2	55.3	1.1	0.3400		
T x R	4	65.3	1.3	0.2850		
Error	18	49.5				
Day 73						
Temperature (T)	2	520.6	7.9	**0.0034**	H with M, L	
Ration (R)	2	78.9	1.4	0.3177		
T x R	4	53.0	0.9	0.4545		
Error	18	56.9				

Table 2.1 Continuation

	df	MS	F	P value	Significant pair-wise tests: Temperature	Significant pair-wise tests: Feeding ration
Gonadosomatic index (dry weight)						
Day 36						
Temperature (T)	2	737.4	12.8	**0.0010**	H with L	
Ration (R)	2	93.4	1.6	0.2300	M with L	
T x R	4	59.2	1.0	0.4060		
Error	18	57.4				
Day 73						
Temperature (T)	2	539.6	7.9	**0.0020**	H with M, L	
Ration (R)	2	91.4	1.3	0.2810		
T x R	4	53.3	0.8	0.5290		
Error	18	68.3				
Connective tissue occupation index						
Day 36						
Temperature (T)	2	656.2	0.9	0.4290		
Ration (R)	2	469.2	0.7	0.5630		
T x R	4	691.2	1.0	0.4330		
Error	18	696.7				
Day 73						
Temperature (T)	2	1719.0	0.9	0.4870		
Ration (R)	2	3562.3	1.9	0.1290		
T x R	4	1287.1	0.7	0.7210		
Error	18	1868.2				
Matured						
Day 36						
Temperature (T)	2	3333.3	2.3	0.1270		
Ration (R)	2	1111.1	0.8	0.4720		
T x R	4	2777.8	1.9	0.1560		
Error	18	1481.5				
Day 73						
Temperature (T)	2	370.4	0.2	0.8500		
Ration (R)	2	10370.4	4.7	**0.0250**		H with M, L
T x R	4	1481.5	0.7	0.6040		
Error	18	2222.2				
Spawned						
Day 73						
Temperature (T)	2	2592.6	0.9	0.4470		
Ration (R)	2	370.4	0.1	0.8780		
T x R	4	2037.0	0.7	0.5850		
Error	18	2963.0				

CHAPTER 3 – Biochemical composition of New Zealand geoduck clam broodstock (*Panopea zelandica*) conditioned under different temperature and feeding regimes

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Abstract

Geoduck clams are amongst the most valuable cultured shellfish available on the world market, driving interest in the establishment of the native New Zealand species, *Panopea zelandica* (Quoy & Gaimard, 1835), as a candidate for aquaculture. A critical element of domestication is the effective management of breeding stock to optimize gamete and offspring quality. To develop a reliable broodstock conditioning protocol for *P. zelandica*, we investigated the biochemical composition of geoducks exposed to 9 factorial treatment combinations of temperature (7.5, 11.5, and 16.5°C) and feeding ration (10,000, 50,000, and 100,000 cells mL^{-1} of a (1:1 cell count) mixture of *Tisochrysis lutea* and *Chaetoceros muelleri*). In addition, a reference group of geoducks was conditioned in an aquaculture pond-nursery system, providing a dilute, mixed phytoplankton culture at ambient temperature. Glycogen, protein, lipid and fatty acid contents of flesh and viscera were measured at days 0, 36 and 73 of conditioning. Glycogen, protein, and lipid analyses indicated that geoducks within all treatments achieved a positive energy balance, except for those in the treatment combining the highest temperature and lowest feeding ration. Comparisons of fatty acid profiles of animals among treatments and with the reference group revealed that eicosapentaenoic (EPA, C20:5n-3), docosahexaenoic (DHA, C22:6n-3) and arachidonic (ARA, C20:4n-6) fatty acids were important contributors to gametogenic development for geoduck conditioning.

3.1 Introduction

An important part of shellfish aquaculture is the identification of optimal biological, physical and chemical parameters to stimulate gonadal maturation, spawning and production of high-quality gametes in broodstock. Commercial hatchery operations require such conditioning protocols to be established and verified (Helm, Bourne & Lovatelli 2004; Utting & Millican 1997). Without this knowledge a reliable and abundant source of larvae cannot be ensured.

Temperature and diet (both quantity and quality) have been identified as important factors regulating bivalve gametogenesis and offspring quality for a variety of species, including the Pacific oyster *Crassostrea gigas* (Caers, Utting, Coutteau, Millican & Sorgeloos 2002; Chávez-Villalba, Pommier, Andriamiseza, Pouvreau, Barret, Cochard & Le Pennec 2002), the clam *Ruditapes philippinarum* (Delgado & Camacho 2007), the mussel *Mytilus galloprovincialis* (Fearman & Moltschaniwskyj 2010) and the Pacific geoduck *Panopea generosa* (Marshall, McKinley & Pearce 2012; Marshall, McKinley & Pearce 2014). However, there may be substantial variation in the optimal conditions required within animals of the same genus and even within animals of the same species from geographically separate populations (Matias, Joaquim, Leitão & Massapina 2009). Furthermore, optimal ranges for some parameters, such as temperature and nutrition, may vary greatly (Ojea, Pazos, Martínez, Novoa, García-Martínez, Sánchez & Abad 2008) or be stable (Martinez & Perez 2003) during gametogenesis, with consequences for spawning success.

For the New Zealand geoduck (*Panopea zelandica*), there is little information regarding the effects of temperature and nutrition on broodstock

conditioning. Attempts to mature *P. zelandica* under temperatures of 8, 12 and 16°C over 2 months resulted in successful maturity at all of these temperatures, but conditioning at 8 or 12°C resulted in higher gonadosomatic indices (Le, Alfaro & King 2014). Gribben, Helson & Jeffs (2004) demonstrated that although gametogenesis in geographically dispersed *P. zelandica* populations began in late autumn, spawning began during spring in one location (Kennedy Bay) and during late summer in another (Shelly Bay). In a related species, *P. generosa*, animals collected from the wild and maintained at 10°C in flowing seawater for one month produced serial weekly spawnings for four months (Beattie 1994). Recently, Marshall *et al.* (2012) found that *P. generosa* could be matured faster and with more oocytes per follicle when conditioned at 7 or 11°C compared to 15 or 19°C. In British Columbia, Canada, Nova Harvest Ltd conditioned *P. generosa* collected from the wild at 12°C and achieved spawning after about 5 weeks, although no other temperatures were trialled for comparison (Dodd 2014). In addition, *P. globosa* became mature after two months when conditioned under a decreasing temperature regime from 22 to 18°C in a semi-open recirculating system (García-Esquivel, Valenzuela-Espinoza, Buitimea, Searcy-Bernal, Anguiano-Beltrán & Ley-Lou 2013). Such studies suggest that geoducks can be induced to mature at temperatures between 7 and 19°C (Beattie 1994; Gribben *et al.* 2004; Marshall *et al.* 2012; Dodd 2014; Le *et al.* 2014).

With regard to the nutritional effects on broodstock conditioning of geoducks, Marshall *et al.* (2014) found no effect of food ration on several indices of reproductive maturity of *P. generosa*, including condition index, gonadosomatic index, connective tissue occupation index or oocyte diameter when fed between

0.8×10^9 and 7.2×10^9 cells of both *Tisochrysis lutea* (formerly known as *Isochrysis affinis galbana* or *T-ISO clone*; Bendif, Probert, Schroeder & de Vargas 2013) and *Chaetoceros muelleri* geoduck^{-1} day^{-1}. In addition, Marshall (2012) did not find any difference in condition index, gonadosomatic index and connective tissue occupation index of *P. generosa* when fed different microalgal species (*T. lutea*, *C. muelleri*, and *Dunaliella tertiolecta*) for 47 days. Furthermore, *P. globosa* could become mature with a sole diet of *T. lutea* at a ration of 16.4×10^9 cells geoduck^{-1} day^{-1} (García-Esquivel *et al.* 2013). However, for *P. zelandica*, more matured geoducks were observed when individuals were fed a mixture of *T. lutea* and *C. muelleri* at 1×10^5 and 5×10^5 cells mL^{-1} compared to 1×10^6 cells mL^{-1} (Le *et al.* 2014). Clearly, further research is needed to establish the optimal feeding requirements of *P. zelandica*. Such investigations will require a deeper understanding of nutritional requirements, including how different nutritional components are accumulated and used to fuel gametogenesis and how these parameters can be manipulated to increase gamete quality.

The gonadal maturation of molluscs is associated with changes in biochemical composition of the animal's tissues, and that gametogenic development is regulated by energy supplies from ingested food and stored reserves (Sastry 1979; Navarro, Iglesias & Larrañaga 1989; Fernández-Reiriz, Pérez-Camacho, Delgado & Labarta 2007). In marine bivalves glycogen stored in the mantle (De Zwaan & Zandee 1972), adductor muscle (Taylor & Venn 1979), and digestive gland (Gabbot 1975) represent the main energy source for gametogenesis. This glycogen source is also responsible for maintenance, especially during winter and periods of low food availability (De Zwaan & Zandee

1972; Gabbot 1975). Lipids tend to accumulate in ovarian tissues during gametogenesis, where they play a vital role in embryonic and larval development (Fearman, Bolch & Moltschaniwskyj 2009). In a study by Gallager & Mann (1986), a significant correlation was found between the egg lipid content and survival of both D-larvae and pediveligers of the clam *Mercenaria mercenaria* and the oyster *Crassostrea virginica*. Thus, since both glycogen and lipids are mobilized to build gametes, and proteins in gametes are synthesized *de novo* (Holland 1978), somatic proteins become energy substrates during sexual maturation (Beninger & Lucas 1984). The utilization of somatic protein has been demonstrated in *R. philippinarum* (Adachi 1979) and *Argopecten irradians concentricus* (Barber & Blake 1981). Lastly, essential fatty acids, such as eicosapentaenoic acid (C20:5n-3), docosahexaenoic acid (C22:6n-3), and arachidonic acid (C20:4n-6) play an important role in gamete development in some species, with significant consequences for subsequent embryo and larval development (Utting & Millican 1998; Fearman *et al.* 2009). For example, a C20:5n-3 supplemented *T. lutea* diet was found to increase the maturation rate, as well as larval and spat survival for the scallop *Nodipecten nodosus* compared with a diet of only *T. lutea* (Sühnel, Lagreze, Zanette, Magalhães & Ferreira 2012).

Recent efforts to domesticate the New Zealand geoduck (*P. zelandica*) have focused on developing reliable broodstock conditioning, larval rearing and juvenile production protocols to sustain this emerging industry. The supply of mature harvested wild geoduck for use as broodstock in New Zealand is unreliable and therefore the ability to hold and condition geoduck is a critical step toward 'on-demand' spawning and the development of a reliable supply of hatchery spat. Wild

P. zelandica are reported to spawn only once a year (Gribben *et al.* 2004), hence conditioning of broodstock under optimal conditions to control spawning time, to achieve multiple spawnings per year, and to synchronize spawning is paramount. Thus, we investigated the reproductive state and maturity of *P. zelandica* broodstock conditioned within different temperature and feeding regimes in a different publication (Le *et al.* 2014). In the present contribution, we report on the biochemical composition of geoducks exposed to different temperature and feeding regimes. The aim is to identify the optimal food and temperature conditions to enhance nutritional state during gametogenesis and achieve maturity. This information will be used to develop a reliable source of gametes to produce hatchery-reared seed for coastal farming.

3.2 Materials and Methods

3.2.1 Animal source

Two-year-old captively-reared geoducks were used for this experiment. The animals were raised from a single spawning of the same set of parents maintained at the Cawthron Institute, Nelson, New Zealand and reared under the same conditions, in sediment-filled tanks supplied with algal pond water (described below). The common history of these animals minimized variability in reproductive condition often associated with season, location, age and genetic make-up. Just prior to the conditioning experiments, geoducks were dug up from the sand where they were grown and immediately cleaned of biofouling. These animals were 57.8 ± 5.5 mm in shell length and 44.8 ± 9.4 g in live weight (mean ± SD, n = 90).

3.2.2 Algal production

Two algal species were used to condition geoducks: *Tisochrysis lutea* and *Chaetoceros muelleri*. Each microalgal species was cultivated by continuous culture in 40 L plastic bags. Pasteurized seawater was pumped continuously to the bags along with Conway growth media. The culture conditions were maintained at 22°C, 100 µE m^{-2} sec^{-1} light intensity, 35 mg L^{-1} salinity, and 4 L min^{-1} aeration with CO_2 addition at 1% v:v. Silicate, in the form of 30 mL of a 30 g L^{-1} stock solution of $Na_2SiO_3.9H_2O$, was continuously added to the *C. muelleri* bags.

3.2.3 Experimental design

The main experiment followed a completely randomized factorial design combining three temperature levels (7.5 ± 0.5, 11.5 ± 0.5, and 16.5 ± 0.5°C) and three feeding rations (10,000, 50,000, and 100,000 cells mL^{-1} of a (1:1 cell count) mixture of *T. lutea* and *C. muelleri*). Nine replicate geoducks were used in each of the 9 treatment combinations. Each geoduck was placed vertically inside a 400 mL bottle, which contained 300 g of fine sand to mimic the natural substrate. Triplicate 10 L PVC tanks per treatment were set up with identical seawater supply, temperature and feeding conditions for that treatment. Three geoducks were randomly placed within each tank. Ideally, each geoduck should have been placed in a separate tank, but space limitations did not allow for use of more tanks. However, each geoduck was contained in a separate bottle and the 9 geoducks were considered as treatment replicates, for a total of 81 geoducks across the experiment. An additional group of 9 geoducks was kept in an aquaculture pond-nursery system supplying a dilute, mixed phytoplankton culture at ambient temperature as a reference group. This pond-nursery system included land-based

seawater ponds, which were enriched with nutrients to promote diatom growth, and maintained on multi-week rotations with regular *ad hoc* top up of fresh seawater. While the pond water contained a mixture of naturally growing microalgae, regular visual inspection of species composition revealed that the dominant species were *Skeletonema costatum* and *Thalassiosira* spp. throughout the duration of the experiment. The pond water temperatures were 10.1 - 14.3°C between days 0 and 36, and 10.4 - 17.6°C between days 36 and 73. Before the start of the experiment, 6 geoducks from the same stock (which had been maintained in the pond-nursery system at about 10°C, as described above) were sacrificed to identify the existing biochemical state and to later compare to the conditioned animals.

To establish the treatments, the animals were first acclimated gradually to 8, 12 and 16°C over a period of three weeks. During this time, they were fed by continuous addition of algae at approx. 40,000 cells mL^{-1} of a 1:1 mixture of *T. lutea* and *C. muelleri*. Salinity was maintained at 33 - 35 mg L^{-1}, oxygen was maintained near saturation by bubbling with air, and pH was 8.0 - 8.2 throughout the experiment. The experimental system included 3 seawater header tanks and 3 feeding header tanks with additional microalgal feed. Manifolds were connected to each header tank to allow the mix of water and microalgae to the experimental tanks continuously at the flow rate of 160 mL min^{-1}.

The mixture of *T. lutea* and *C. muelleri* was pumped pneumatically (using PB1000 series pumps, SMC Pneumatics, Indianapolis, USA) into the feeding header tanks at different rates to achieve the desired feed ration for each treatment. One geoduck from each of the replicate tanks was randomly removed

and sacrificed on day 36 and on day 73, resulting in three random geoducks from each treatment.

3.2.4 Biochemical analyses

Sample preparation

Biochemical analyses were performed on 6 geoducks at the start (day 0), on 3 geoducks from each treatment and the reference group collected at day 36, and on another 3 geoducks per treatment and the reference group at day 73. The animals were first dissected to separate flesh, viscera tissue and shell. Flesh tissue included siphon, adductor muscles, thin mantle, and muscular mantle. Visceral tissue included gonad, stomach, intestine, style sac and digestive gland, as well as the heart, kidney, foot, labial palps and gills. It must be noted that the gonad and other organs are not readily separated from the rest of the visceral mass. The entire visceral mass was used in the analysis. Shell lengths were measured to the nearest 0.1 mm. Wet weights of the whole animal, the shell, viscera and flesh tissues were determined to the nearest 0.1 g. The viscera and flesh tissues were snap frozen and stored separately at - 80°C until analysis. The dry weights of the shells were obtained by drying at 104°C for 24 h. The tissues were freeze-dried and dry weights obtained to the nearest 0.0001 g. All tissue samples were then ground to powder, and sub-sampled into three aliquots for glycogen, protein, lipid or fatty acid analysis.

Proximate analyses

The procedure for the fractionation of the homogenate for glycogen and protein analysis was conducted following Holland & Hannant (1973). Briefly, about 10 mg of freeze-dried, powdered sample was mixed with 500 µL distilled deionized water

in a glass tube by vortex and sonication for 5 min. Then, 250 µL of cold 15% trichloroacetic acid (TCA) was added and mixed by shaking for 5 min. After standing at 4°C for 10 min the tube was then centrifuged at 800 *g* for 10 min. The supernatant was collected and the precipitate was extracted again as above, but with 500 µL 5% TCA. The two supernatants were combined and diluted 15-fold with 10% TCA for glycogen analysis using an iodine assay (Dreiling, Brown, Casale & Kelly 1987). A bovine liver glycogen standard (Sigma Aldrich) and an iodine color reagent were prepared as described in Dreiling *et al.* (1987) and absorbance was measured at 460 nm after 20 min incubation at room temperature.

The TCA precipitated pellet was dissolved in 1.25 mL 1N NaOH at 56°C for 30 min for protein determination. Protein content was determined on samples diluted 15 fold with 1N NaOH, using a Pierce Micro BCA Protein Assay Kit according to the manufacturer's instructions.

Total lipid content was determined gravimetrically following Bligh & Dyer (1959). About 100 mg of powdered sample was mixed with 400 µL cold distilled deionized water, 500 µL chloroform, and 1000 µL methanol. After sonication in a bath for 10 min at 4°C 500 µL chloroform was added. The sample was vortexed and sonicated again for 5 min, followed by the addition of 500 µL distilled deionized water before vortexing and sonicating for another 5 min. The sample was then centrifuged at 3000 *g* for 5 min and the lower phase removed with a glass Pasteur pipette and placed into a pre-weighed glass tube. Another 500 µL of chloroform was added to the upper phase to re-extract the residue, followed by vortexing, sonicating, centrifuging, and pipetting as above. The combined extract was dried by nitrogen gas before being weighed.

Fatty acid analyses

Fatty acid methyl esters were obtained following De La Cruz-Garcia, López-Hernández, González-Castro, Rodríguez-Bernaldo & Simal-Lozano (2000). A sample of 12 ± 0.1 mg was placed in a 10 mL test tube. A 10 µL volume of a 2 g L^{-1} solution of tridecanoic acid in toluene was added as an internal standard before a further 490 µL of toluene and 750 µL of freshly prepared 5% methanolic HCl were added. The mixture was vortexed before the headspace of each tube was filled with nitrogen, sealed and placed in a water bath at 70°C for 2 h. Tubes were cooled to room temperature before adding 1 mL of 6% aqueous K_2CO_3 and 500 µL of toluene and vortexing gently to mix. The mixture was centrifuged at 1100 *g* for 5 min and the organic phase was removed with a glass Pasteur pipette for analysis of Fatty Acid Methyl Esters (FAME). FAMEs were separated and quantified on a Shimadzu GC2010-Plus GLC with a Flame Ionisation Detector (FID), a split injector and an AOC-20i auto-injector. The column was a Phenomenex Zebron ZB-WAX capillary measuring 0.25 mm x 30 m x 0.25 µm. The beginning oven temperature was 140°C, rising to 245°C at a rate of 5°C per min and held for 15 min. The carrier gas was nitrogen at a flow rate of 60 mL min^{-1} and a linear velocity of 20 cm s^{-1}. Detector and injector were at 250°C and the split ratio was 50:50. FAME peaks in samples were identified and quantified by comparison with serial dilutions of a 37 FAME standard (Supelco product 47885-U; Sigma Aldrich, Sydney, Australia) and normalised to the area of the internal standard.

3.2.5 Statistical analyses

Due to the low number of animals available for this experiment, non-parametric multivariate analysis of variance based on permutations (PERMANOVA) was used to test the main effects (temperature and feeding ration) on glycogen, protein, and lipid contents. Two-way PERMANOVA was conducted on Bray–Curtis distances with 999 permutations calculated from raw data and standardized data. By using permutations, the tests did not need specific assumptions concerning the number of variables or the nature of their individual distributions or correlations. If the main effects and/or interactions were significant, pair-wise comparisons were conducted by Tukey tests. All statistical comparisons were made with a level of significance of $\alpha \geq 0.05$.

Cluster analysis and non-metric multidimensional scaling (nMDS) were used to identify differences in fatty acid profiles among treatments. Prior to analysis, the fatty acid data were standardized as percentage of total fatty acids and arcsine square root transformed. The Bray-Curtis measurement of similarity was used for both classification and ordination. In order to identify which fatty acids were responsible for separations between treatment and reference groups, principal component analysis (PCA) was applied to the standardized data set after auto-scaling (mean-centred and divided by the standard deviation of each variable). The loading values for the most important fatty acids in the PCA were obtained. The percentage contribution of each fatty acid to the difference between treatment and reference groups was quantified by SIMPER analysis with the cut off for low contributions at 90%. PERMANOVA, cluster, and n-MDS analyses were

performed with PRIMER Version 6.1. PCA analyses were performed with MetaboAnalyst Version 3.0.

3.3 Results

3.3.1 Glycogen

For the flesh samples, mean±SD glycogen values were 304.0 ± 41.4 mg g^{-1} dry weight for the initial group and 400.9 ± 38.8 and 333.3 ± 37.6 mg g^{-1} dry weight for the reference groups at 36 and 73 days, respectively (Fig. 3.1). Conversely, the pooled mean ±SD glycogen values for the temperature-ration treatments were 293.6 ± 49.6 and 250.3 ± 60.0 mg g^{-1} dry weight at 36 and 73 days, respectively. For the viscera samples, the mean ±SD glycogen content for the initial group was 351.2 ± 51.5 mg g^{-1} dry weight, and the reference group had values of 258.2 ± 97.7 and 278.9 ± 53.9 mg g^{-1} dry weight at 36 and 73 days, respectively. The pooled mean ±SD glycogen values for the temperature-ration treatments were 277.2 ± 58.6 and 248.5 ± 42.2 mg g^{-1} dry weight at 36 and 73 days, respectively.

At day 36, feeding ration and the interaction of temperature and feeding ration had a statistically significant influence on both flesh and viscera glycogen (Table 3.1). However, these differences were not observed on day 73, where temperature and the interaction were significant for flesh samples only. The significant feeding ration patterns for glycogen contents at day 36 are complex. The flesh samples of animals fed low rations show a slight increase in glycogen content with increasing temperature, but for animals fed medium and high rations, the glycogen contents generally decreased with increasing temperature. The opposite trends were observed for viscera samples.

3.3.2 Protein

Protein contents were similar for geoducks across temperature-ration treatments compared to the initial and reference groups, except for a slightly higher protein content in the viscera of geoducks within the initial and reference groups compared to the treatments (Fig. 3.1). For flesh samples, the initial group had protein contents (mean ±SD) of 217.2 ± 32.9 mg g^{-1} dry weight, while the reference group had mean ±SD values of 224.7 ± 29.9 and 237.2 ± 13.5 mg g^{-1} dry weight on day 36 and 73, respectively. Combined protein mean ±SD values for temperature-ration treatments were 215.9 ± 24.5 and 202.0 ± 34.7 mg g^{-1} dry weight on day 36 and 73, respectively. For viscera samples the mean ±SD values were 235.6 ± 35.0 mg g^{-1} dry weight for the initial group and 231.2 ± 30.0 and 227.4 ± 46.6 mg g^{-1} dry weight for the reference group on day 36 and 73, respectively. Conversely, temperature-ration treatments had the pooled mean (±SD) values 186.2 ± 34.3 mg g^{-1} dry weight on day 36 and 199.0 ± 25.7 mg g^{-1} dry weight on day 73.

Temperature had a significant effect on protein contents in both flesh and viscera samples at day 36 and on flesh at day 73 (Fig. 3.1, Table 3.1). Protein contents were lower for flesh samples in geoducks exposed to medium temperatures compared to low and high temperatures, and the opposite trend holds for viscera samples. In addition, protein contents on viscera samples on day 36 were significantly lower for medium food rations across temperature compared to low and high food rations. However, these differences were not observed on day 73. Significant interactions of main effects were encountered in analyses of flesh and viscera samples on day 36 (Table 3.1), indicating that temperature did not have the same effect among food rations.

3.3.3 Lipids

Lipid contents were lower for flesh samples compared to viscera samples for all geoducks (initial, treatment and reference groups) for all sampling dates (Fig. 3.1; $P < 0.001$). The initial group had slightly lower lipid values compared to all treatment and reference groups in flesh samples, and the opposite trend was observed for viscera samples (Fig. 3.1). For flesh samples, the mean ±SD lipid contents for the initial group were 41.5 ± 4.9 mg g^{-1} dry weight, while the pooled mean of the temperature-ration treatments were 55.4 ± 12.6 mg g^{-1} dry weight on day 36 and 49.5 ± 8.0 mg g^{-1} dry weight on day 73, and the values for the reference group were 51.5 ± 8.8 and 53.7 ± 2.0 mg g^{-1} dry weight on days 36 and 73, respectively. Conversely, viscera samples for the initial group had a mean ± SD lipid content of 125.2 ± 13.3 mg g^{-1} dry weight, while the pooled values for the temperature-ration treatments on days 36 and 73 were 96.9 ± 11.5 and 100.3 ± 16.6 mg g^{-1} dry weight, respectively. Lipid content values for the reference groups were 117.7 ± 17.6 and 98.8 ± 4.5 mg g^{-1} dry weight for days 36 and 73, respectively. Food ration had a significant effect on lipid content of viscera on both days 36 and 73, with a general trend of increasing lipid content with increasing food ration (Fig. 3.1, Table 3.1). Temperature also had a significant effect on lipid in viscera, but only on day 36. There were no significant effects of ration or temperature on flesh lipids (Table 3.1).

3.3.4 Fatty acids

Multivariate analyses of fatty acid profiles of flesh samples did not detect any differences (95% similarity) among geoducks from different treatments (Fig. 3.2) or between treatments and reference group throughout the experiment (Fig. 3.3). The

mean (±SD) percentage for saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) proportions of flesh samples within the initial group were 28.97 ± 1.08%, 17.38 ± 1.98%, and 54.51 ± 2.90%, respectively (Supplementary Table 3.1). For the temperature-food ration treatments, the pooled mean (±SD) SFA, MUFA, and PUFA proportions within flesh samples were 29.2 ± 1.2%, 18.7 ± 1.1%, and 52.5 ± 2.7%, respectively on day 36, and 29.7 ± 1.2%, 18.5 ± 1.9%, and 51.8 ± 2.8%, respectively on day 73 (Supplementary Tables 3.2&3.3). Flesh samples of animals within the reference group had mean (±SD) SFA, MUFA, and PUFA proportions of 28.6 ± 1.0%, 19.1 ± 0.1%, and 52.3 ± 1.2%, respectively on day 36, and 28.0 ± 0.8%, 17.7 ± 0.2%, and 54.3 ± 0.9%, respectively on day 73 (Supplementary Tables 3.2&3.3).

Results for fatty acid analysis from the viscera samples also revealed no differences among geoducks within treatments on day 36 and 73 (Figs. 3.2&3.3, Supplementary Tables 3.4&3.5). However, clear differences were observed in fatty acid profiles between the temperature-ration treatment viscera samples and those in the reference group (Fig. 3.2&3.3, Table 3.2). For viscera samples, the initial mean (±SD) SFA, MUFA, and PUFA proportions were 27.6 ± 1.4%, 23.7 ± 2.2%, and 48.7 ± 0.9%, respectively (Supplementary Table 3.1). The pooled mean (±SD) SFA, MUFA, and PUFA proportions of viscera samples across treatments were 28.5 ± 0.6%, 20.9 ± 2.2%, and 51.3 ± 1.3%, respectively on day 36, and 29.0 ± 0.6%, 20.1 ± 1.0%, and 51.0 ± 1.3%, respectively on day 73 (Supplementary Tables 3.4&3.5). For the reference group mean (±SD) SFA, MUFA, and PUFA proportions were 28.6 ± 0.7%, 22.9 ± 1.8%, and 48.5 ± 2.0%, respectively on day

36, and 29.6 ± 1.0%, 22.6 ± 0.4%, and 47.8 ± 1.2%, respectively on day 73 (Supplementary Tables 3.4&3.5).

A principal components analysis (PCA) of fatty acids from viscera samples indicated clear separation between samples from the reference group and those of treatments, for both day 36 and day 73 (Fig. 3.3). This separation was mostly observed in the first principal component (PC1); the eigenvector coefficients are plotted in Fig. 3.3. For viscera samples from day 36, eicosapentaenoic acid (C20:5n-3), elaidic acid (C18:1n-9t), and palmitolenic acid (C16:1n-7) were the most dominant fatty acids for the reference group, and linoleic acid (C18:2n-6), docosahexaenoic acid (C22:6n-3), arachidonic acid (C20:4n-6), oleic acid (C18:1n-9c), and linolenic acid (C18:3n-3) were most dominant for samples within the treatments. Furthermore, SIMPER analyses also indicated that C22:6n-3, C20:5n-3 and C20:4n-6 contributed the most to the difference between the two groups with 15.8%, 10.1%, and 12.5%, respectively (Table 3.2). For viscera day 73 samples, the dominant fatty acids for the reference group were C14:0, C16:0, C16:1n-7 and C20:5n-3, while those for the treatments were C18:0, C18:1n-9c, C20:4n-6 and C22:6n-3. SIMPER results showed that C22:6n-3, C20:5n-3 and C20:4n-6 contributed only 10.3%, 11.3% and 8.9% to this analysis, while C16:1n-7 and C18:1n9-t contributed 15.2% and 15.4%, respectively (Table 3.2).

3.4 Discussion

The storage and utilization of nutrients in adult bivalves is known to vary with the reproductive cycle and available food supplies (Gabbott 1975). During reproductive conditioning and maturity, these nutrients are remobilized and allocated to the production of gametes. Thus, changes in the localization of

compounds between different tissues may be used as indicators of conditioning under different environmental and/or culturing conditions. Given that over 50% of geoducks in the treatment groups achieved maturity and those in the initial group were all immature (Le *et al.* 2014), we would have expected to see a significant reduction in glycogen from day 0 onward. A decrease in glycogen has been considered to be an indication of gametogenic development (Walne 1970; De Zwaan & Zandee 1972; Berthelin, Kellner & Mathieu 2000; Ojea, Pazos, Martínez, Novoa, Sánchez & Abad 2004; González-Araya, Quéau, Quéré, Moal & Robert 2011). Indeed, glycogen values in viscera were slightly lower in geoducks in the treatment groups compared to those in the initial group. A decrease in glycogen in viscera has been observed in the blue mussel *Mytilus edulis* during gametogenesis in mid-winter periods (De Zwaan & Zandee 1972), and stored glycogen has been reported to be used up during gonad development in the European flat oyster *Ostrea edulis* (Walne 1970; González-Araya *et al.* 2011). However, no such differences were observed between groups in flesh samples, except for animals exposed to high temperature and low food ration at day 73. The observed reduction in flesh glycogen reserves in the high temperature-low food ration group suggests that flesh glycogen was used to sustain the high metabolic demands. Similarly, glycogen in the adductor muscle has been shown to provide metabolic energy for the scallop *A. irradians concentricus* (Barber & Blake 1981). There was a decreasing trend in glycogen content in flesh samples when temperature increased, at least for medium and high feeding rations, while the opposite was the case for viscera samples, which contained the gonads. Thus, these data suggest the possibility that glycogen reserves in viscera were used to develop gonad

material, while flesh glycogen was utilized to maintain metabolic demands. The combined results of the present study and the reproductive study by Le *et al.* (2014) indicate that glycogen contents were sufficient to support gonad development in animals across all temperature and feeding regimes, except for those animals exposed to high temperature and low food rations on day 73, as observed by a dramatic decrease in glycogen in flesh samples of these animals. These results suggest that these extreme conditions were too stressful to maintain positive energy balance (Delgado & Camacho 2007). This conclusion is supported by the comparable trends in condition and gonadosomatic indices reported for these animals by Le *et al.* (2014). Similarly, the Manila clam *R. philippinarum* showed depleted glycogen contents when reared under low food rations and high temperatures (Fernández-Reiriz *et al.* 2007).

Protein contents in flesh samples of geoducks conditioned in different temperature and feeding ration regimes were similar to those of animals in the initial and reference groups, but viscera samples of animals within treatments had slightly lower protein contents than those in the initial and reference groups. Normally, protein content is expected to increase in the gonads during early gametogenesis, and then rapidly decrease as protein contributes to the formation of gametes, especially sperm (Benomar, Costil, El Filali, Mathieu & Moukrim 2010; Li, Osada & Mori 2000, Li, Liu, Shirasu, Chen & Jiang 2006; Li, Yang, Ke & Kong 2011). Thus, the differences in viscera samples are interpreted to reflect the increasing gonadal development of treatment animals compared to those in the initial and reference groups, which did not mature (Le *et al.* 2014). Similarly, high protein contents have been found in the gonad-viscera mass when animals are in

early gametogenesis, followed by a decrease in protein content until the end of gametogenesis in the clam *Ruditapes decussatus* (Ojea *et al.* 2004) and the surf clam *Mactra veneriformis* (Ke & Li 2013). In the present study, 97.9% (94/96) of the experimental geoducks were male as this species likely changes sex from male to female in adulthood (Gribben & Creese 2003). The two females did not separate from the males in the nMDS plot and there was no significant difference in the gonadosomatic index values between male and female (Le *et al.* 2014). Therefore, using all geoducks in this analysis, results showed increased spermatogenesis within treatment individuals compared to those in the initial and reference groups. The same trend was found in the male gonad of the mussel *Perna perna* (Benomar *et al.* 2010) the clam *Mactra chinensis* (Li *et al.* 2011), and the oysters *Crassostrea gigas* (Li *et al.* 2000) and *C. plicatula* (Li *et al.* 2006). The mechanism for this reproductive process was described by Unuma, Yamamoto, Akiyama, Shiraishi & Ohta (2003) in three urchin species as the use of protein stored in testicular nutritive phagocytes for synthesis of nucleic acids that constitute sperm.

It has been observed that protein reserves in other tissues, such as the adductor muscle, are not utilized for gametogenesis unless a critical starvation or stress point is experienced (Beninger & Lucas 1984, Ruiz, Abad, Sedano, Garcia-Martin & Sanschez-Lospez 1992, Ojea *et al.* 2004; Ke & Li 2013). Thus, the fact that the protein levels in the flesh of combined treatment animals remained similar relative to the initial and reference groups indicates that geoducks were not food limited during this experiment. However, temperature was found to have a significant effect on protein content in both flesh and viscera samples among treatment groups. The generally opposite patterns of protein content with different

temperatures observed between flesh and viscera samples suggest that protein may have been mobilized from one area to the other during conditioning. However, more specific studies targeted on the investigation of biochemical processes involved in protein utilization and mobilization would be needed to elucidate this pattern.

Significantly greater lipid contents were observed in viscera samples compared to flesh samples for all animals, including the initial and reference groups and treatments throughout the experimental period. The digestive gland and the gonad, within the visceral mass, are known to be the main lipid storage places in bivalves, such as the scallop *A. irradians concentricus* (Barber & Blake 1981), the oyster *C. gigas* (Allen & Conley 1982), the clam *R. decussatus* (Ojea *et al.* 2004), and the surf clam *M. veneriformis* (Ke & Li 2013). Thus, it is suggested that, in the present study, lipid storage was taking place in storage cells within the viscera of geoducks as gametogenesis was taking place.

Although muscular cells within the flesh are not known to store large quantities of lipids (Mathieu & Lubet 1993), the higher lipid content in flesh samples of treatment and reference group animals compared to those in the initial group indicates that some lipid storage (for energy reserves) took place in muscle tissues. This indicates that food was not limiting during the experimental period. This lipid storage in muscle tissues during positive energy balance has been shown for other bivalves, such as the clams *R. decussatus* (Pérez-Camacho, Delgado, Fernández-Reiriz & Labarta 2003) and *R. philippinarum* (Fernández-Reiriz *et al.* 2007). For the case of the lower lipid contents in viscera samples of treatment and reference group animals compared to those in the initial group, it is

suggested that the ingested lipids were used to fuel gametogenesis (treatment animals) and/or remobilization (treatment and reference group) to muscle flesh tissues for storage. Utilization of lipids from the digestive gland for gametogenesis has been demonstrated for several bivalve species, including *Chlamys hericia* (Vassallo 1973), *A. irradians concentricus* (Barber & Blake 1981) and *A. ventricosus* (Ruiz-Verdugo, Racotta & Ibarra 2001). During abundant food supply conditions, mobilization of lipids has been shown to take place from the digestive glands to muscle tissues, but not the other way around (Allen & Conley 1981; Mathieu & Lubet 1993).

The quantity and quality of microalgal feeds, especially with regards to their fatty acid composition, are known to have a direct influence on bivalve nutrition (Utting & Millican 1997). In this study, treatment geoducks were fed different concentrations of *T. lutea* and *C. muelleri* at various temperatures. However, no differences in fatty acid profiles were found in flesh or viscera samples among animals from different experimental treatments over time. As geoducks were all given the same mix of fatty acids in the same proportions, no differences among treatments were expected. In addition, flesh and viscera samples from initial and reference (pond) groups had similar fatty acid profiles (data not shown). This indicates that the fatty acid composition of the algal species available in the pond culture did not change enough to have a significant impact on the tissue composition of the geoducks over 73 days.

In contrast, clear differences were observed in viscera samples between treatment animals and those in the initial and reference groups. These results reflect the differences in fatty acid composition between the cultivated

monocultures of *T. lutea* and *C. muelleri* provided to the treatment animals and the diet provided to the initial and reference groups, which was a mixed microalgal diet naturally growing in the pond water (contained a dominance of *S. costatum* and *Thalassiosira* spp.). The microalgal feed produced at Cawthron Institute contained 4.8% C20:4n-6 in *C. muelleri* and 9.9% C22:6n-3 in *T. lutea* (Adams, Salinas-Flores & Lim 2013), while the two main species (*S. costatum* and *Thalassiosira* spp.) found in the pond water might be low in both C20:4n-6 and C22:6n-3 (Volkman, Jeffrey, Nichols, Rogers & Garland 1989). Deficiencies in C22:6n-3 have been shown to result in poor conditioning in other bivalves, such as the clam *Donax trunculus* (Martínez-Pita, Hachero-Cruzado, Sánchez-Lazo & Moreno 2012) and the scallop *Pecten maximus* (Soudant, Moal, Marty & Samain 1996). In addition, reproductive maturation has been shown to be attained with high proportions of C22:6n-3 and C20:4n-6 (Baptista, Repolho, Maulvault, Lopes, Narciso, Marques, Bandarra & Rosa 2014). Thus, C22:6n-3 is associated with oogenesis and embryogenesis (Ehteshami, Christianus, Rameshi, Harmin & Saad 2011), where it plays a major role in the structure and function of cell membranes involved in these reproductive processes (Utting & Millican 1998; Soudant *et al.* 1996). Similarly, C20:4n-6 is incorporated into oocytes during maturation (Marty, Delaunay, Moal & Samain 1992; Soudant *et al.* 1996), and is a precursor of eicosanoids, including prostaglandins, which influence reproductive development in bivalves (Hurtado, Reza, Ibarra, Wille, Sorgeloos, Soudant & Palacios 2009). It should be noted that the reference animals in our study became mature two months after the end of the experiment (Le *et al.* 2014), when *Chaetoceros constrictus*, which contains high levels of C20:4n-6 (Zhukova & Aizdaicher 1995),

was the dominant species in the pond water (W. Hiini & R. Knecht, pers. comm.). These results indicate that *T. lutea* and *C. muelleri* are sufficient to condition geoducks under the feeding rations used in this experiment. Indeed, these algal species are commonly used in aquaculture production because they have a wide range of essential fatty acids suitable for both growth and reproduction of shellfish species (Helm *et al.* 2004).

Temperature is well-known to determine growth in bivalves. However, the effect of temperature on biochemical processes, such as fatty acid storage and utilization is not well understood. In this study, fatty acid profiles in flesh samples did not differ between the initial and reference groups (data not shown), and among the treatments. Similarly, fatty acid profiles of total phospholipids in foot tissues of *M. edulis* individuals, previously adapted to different temperatures, were shown to remain constant (Chebotareva, Zabelinskii, Shukolyukova & Krivchenko 2010). In fish, temperature did not have significant impact on fatty acid composition in muscle of juvenile Pacific cod *Gadus microcephalus* and walleye Pollock *Theragra chalcogramma* over a 2-month period (Copeman, Laurel & Parrish 2013), and juvenile black bream *Acanthopagrus buthcheri* (Elsdon 2010). Thus, it is suggested that the thermal influence in fatty acids of muscular flesh is minimal within a temperature range of 7.5-16.5°C, over a 2 month period.

In conclusion, this is the first study to investigate biochemical processes involved in broodstock conditioning of the New Zealand geoduck (*P. zelandica*). The results of analyses of proximate composition of geoducks exposed to different water temperatures and food ration regimes indicate that all treatments resulted in positive energy balance for long-term conditioning of this species, except for the

extreme condition of high temperature (16-17°C) and low food ration (10,000 cells mL^{-1} of a 1:1 mixture of *T. lutea* and *C. muelleri*), which appeared to result in a negative energy balance. The fatty acid analyses herein and histological observations reported in Le *et al.* (2014) corroborate the fact that gonad maturation occurred in animals within all treatment conditions but was absent in the initial group and did not occur in the reference (nursery system) groups even after 72 days. Thus, from a practical and cost-effective point of view, a medium (11-12°C) or low (7-8°C) temperature regime with medium feeding rations of 50,000 cells mL^{-1} of a 1:1 cell count mixture of *T. lutea* and *C. muelleri* would be sufficient to condition *P. zelandica* broodstock. The New Zealand geoduck is an emerging aquaculture species with great potential for future export markets. With this in mind, the present contribution provides the first description of biochemical effects of different broodstock conditioning regimes, providing the first steps towards securing reliable production of seed for on-growing.

3.5 References

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Figure 3.1 Proximate composition (glycogen, protein and lipids) of geoduck flesh and viscera at initial stocking (day 0; n = 6), and in the 9 treatment (temperature and feeding ration combinations; n = 3 individuals per treatment) and reference groups (R; n = 6) on days 36 and 73. Data are expressed as mean ± SD (mg g^{-1} dry weight). Temperature levels are denoted as (L = low, 7.5 ± 0.5°C; M = medium, 11 ± 0.5°C; H = high, 16 ± 0.5°C) and feeding rations are denoted as (L = low, 10,000 cells mL^{-1}; M = medium, 50,000 cells mL^{-1}; H = high, 100,000 cells mL^{-1}). The treatments are labelled as temperature-ration combinations (e.g., H-L = high temperature-low ration).

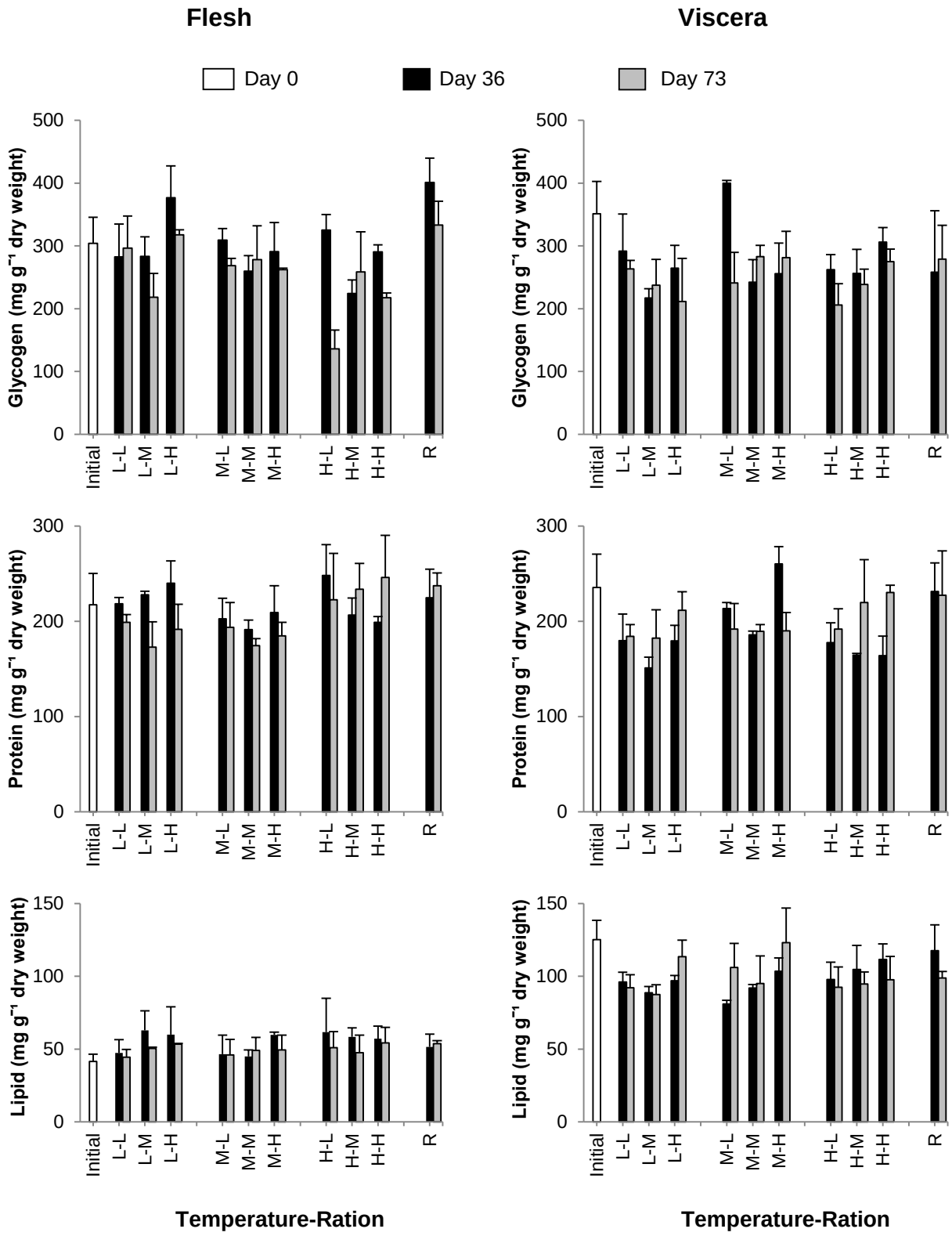
Flesh
Viscera
Day 0
Day 36
Day 73
Glycogen (mg g⁻¹ dry weight)
Protein (mg g⁻¹ dry weight)
Lipid (mg g⁻¹ dry weight)
0
100
200
300
400
500
50
150
Initial
L-L
L-M
L-H
M-L
M-M
M-H
H-L
H-M
H-H
R
Temperature-Ration

Figure 3.2 n-MDS plots of fatty acid profiles of geoduck flesh and viscera in different treatments (temperature and feeding ration combinations) and reference group on days 36 and 73. Clusters are represented at 95% similarity. Vectors represent Pearson correlations for individual fatty acids. Treatment and reference groups are denoted as in Figure 3.1.

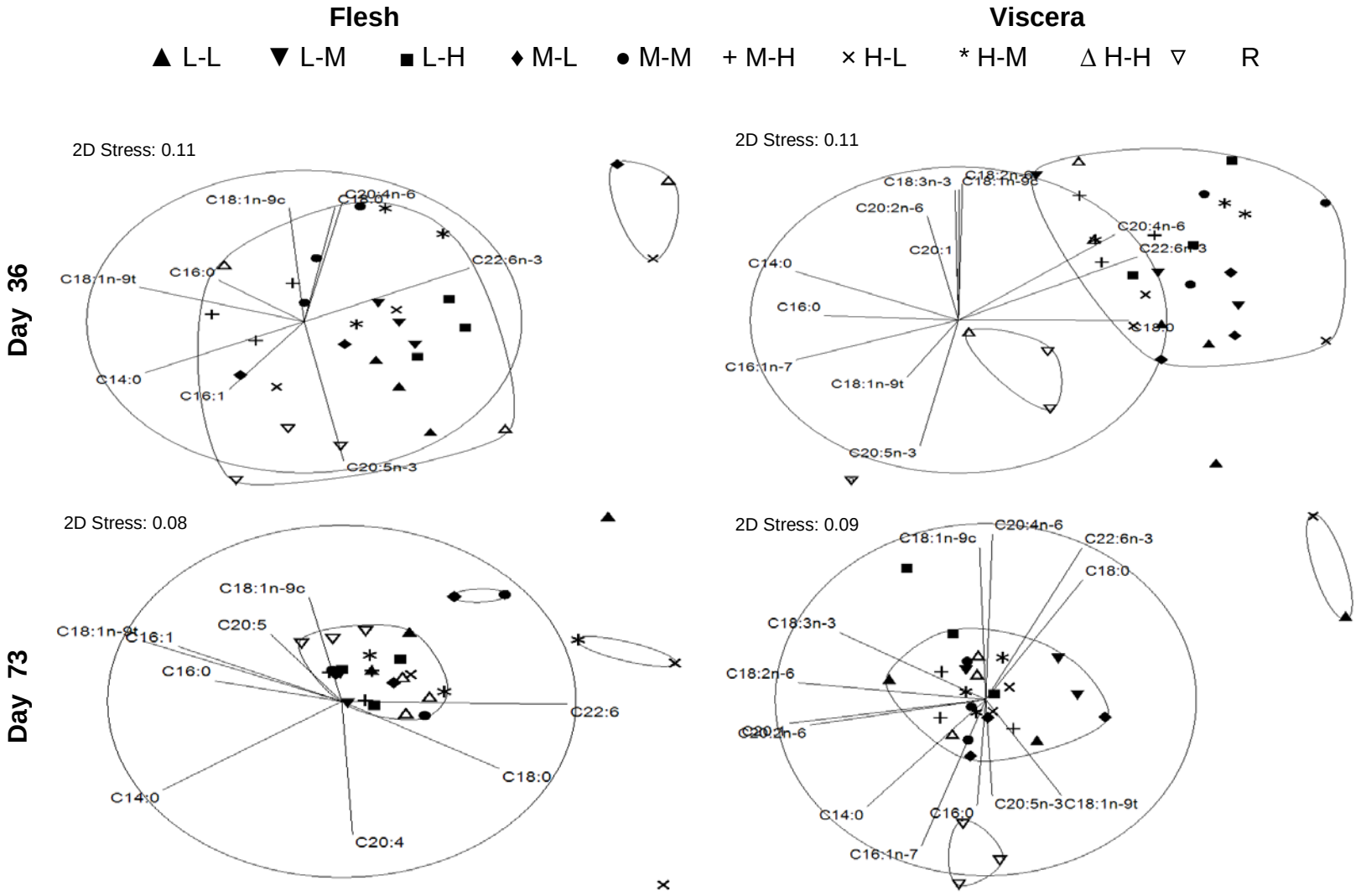
Flesh
Viscera
▲ L-L ▼ L-M ■ L-H ♦ M-L ● M-M + M-H × H-L * H-M ∆ H-H ▽ R
Day 36
Day 73
2D Stress: 0.11
2D Stress: 0.11
2D Stress: 0.08
2D Stress: 0.09
C18:1n-9c
C20:4n-6
C18:0
C22:6n-3
C18:1n-9t
C16:0
C14:0
C16:1
C20:5n-3
C18:3n-3
C18:2n-6
C20:2n-6
C20:1
C16:1n-7
C20:5
C22:6
C20:4

Figure 3.3 PCA plots of flesh and viscera samples from geoducks in different treatments (temperature and feeding ration combinations; light circles) and reference groups (dark circles) on days 36 and 73. Clusters represent 95% confidence regions. PC1 eigenvalues for fatty acids contributing to the separation of treatment and reference groups.

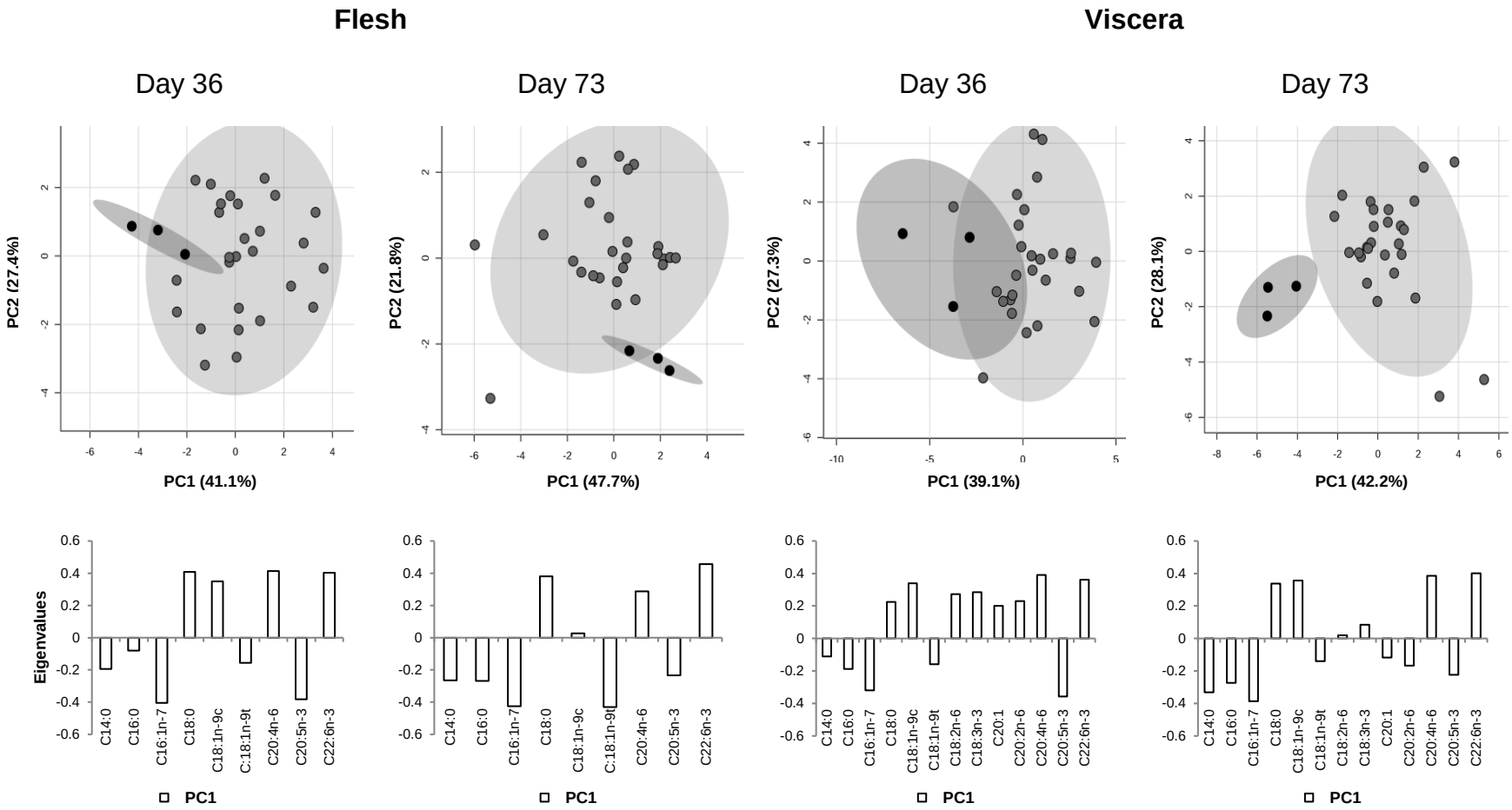
Flesh
Viscera
Day 36
Day 73
Day 36
Day 73
PC2 (27.4%)
PC1 (41.1%)
PC2 (21.8%)
PC1 (47.7%)
PC2 (27.3%)
PC1 (39.1%)
PC2 (28.1%)
PC1 (42.2%)
Eigenvalues
C14:0
C16:0
C16:1n-7
C18:0
C18:1n-9c
C:18:1n-9t
C18:1n-9t
C20:4n-6
C20:5n-3
C22:6n-3
C18:2n-6
C18:3n-3
C20:1
C20:2n-6
PC1

Table 3.1 PERMANOVA results for proximate composition of flesh and viscera samples for days 36 and 73. Significant factors and interactions are in bold. Significant pairwise comparisons for main effects are indicated.

		Flesh				Viscera		
Source	**df**	**MS**	**F**	***P***	**df**	**MS**	**F**	***P***
Glycogen								
Day 36								
Temperature (T)	2	71.9	2.2	0.119	2	97.9	2.2	0.128
Ration (R)	2	291.1	8.9	**0.003**	2	395.9	8.9	**0.002**
T x R	4	117.2	3.6	**0.023**	4	192.7	4.3	**0.011**
Error	18	32.6			18	44.6		
Significant pair-wise tests	R:	LxM, MxH			R:	LxM		
Day 73								
Temperature (T)	2	715.2	12.8	**0.001**	2	108.9	1.7	0.193
Ration (R)	2	173.4	3.1	0.061	2	38.3	0.6	0.549
T x R	4	438.4	7.8	**0.001**	4	152.6	2.4	0.088
Error	18	55.9			18	62.8		
Significant pair-wise tests	T:	LxH, MxH						
Protein								
Day 36								
Temperature (T)	2	97.2	5.2	**0.021**	2	471.6	23.2	**0.001**
Ration (R)	2	22.1	1.2	0.327	2	164.7	8.1	**0.002**
T x R	4	52.4	2.8	0.065	4	65.3	3.2	**0.034**
Error	18	18.8			18	20.3		
Significant pair-wise tests	T:	LxM			T:	LxM, MxH		
					R:	LxM, MxH		
Day 73								
Temperature (T)	2	362.4	7.7	**0.005**	2	83.8	2.5	0.094
Ration (R)	2	30.2	0.6	0.532	2	64.9	1.9	0.185
T x R	4	25.3	0.5	0.703	4	27.4	0.8	0.520
Error	18	46.9			18	34.0		
Significant pair-wise tests	T:	LxH, MxH						
Lipid								
Day 36								
Temperature (T)	2	129.2	0.9	0.425	2	101.9	5.7	**0.009**
Ration (R)	2	132.5	1.0	0.394	2	98.7	5.5	**0.016**
T x R	4	115.8	0.8	0.503	4	31.7	1.8	0.176
Error	18	137.7			18	18.0		
Significant pair-wise tests					T:	MxH		
					R:	LxH, MxH		
Day 73								
Temperature (T)	2	17.7	0.2	0.821	2	85.5	1.7	0.196
Ration (R)	2	67.1	0.8	0.441	2	191.4	3.8	**0.041**
T x R	4	25.4	0.3	0.852	4	36.3	0.7	0.571
Error	18	80.4			18	50.2		
Significant pair-wise tests					R:	MxH		

Table 3.2 SIMPER results indicating the percentage contribution of different fatty acids to the difference in treatment samples (flesh & viscera) with respect to the reference group. ND denotes values not detected.

Fatty acid	Flesh day 36	Flesh day 73	Viscera day 36	Viscera day 73
C14:0	5.98	7.14	4.26	6.57
C16:0	ND	6.4	5.52	6.86
C16:1n-7	11.21	8.43	9.68	15.22
C18:0	12.91	16.11	5.95	4.77
C18:1n-9c	11.78	15.36	7.6	ND
C18:1n-9t	6.25	ND	4.86	15.4
C18:2n-6	ND	ND	8.49	5.41
C18:3n-3	ND	ND	9.29	7.11
C20:4n-6	18.6	6.88	12.46	8.86
C20:5n-3	16.92	27.43	10.1	11.34
C22:6n-3	11.61	6.92	15.84	10.27

Supplementary Table 3.1. Fatty acid profiles of *T. lutea*, *C. muelleri* from Adams et al. (2013), and fatty acid profiles of flesh and viscera samples of geoducks within the initial group (day 0). Data are expressed as mean (±SD). ND denotes values not detected.

Fatty acid	*T. lutea*	*C. muelleri*	Flesh day 0	Viscera day 0
C14:0	16.5	16.9	3.05(0.38)	3.13(0.23)
C15:0	ND	0.5	ND	ND
C16:0	9.4	10.6	16.54(0.47)	16.60(0.81)
C18:0	1.1	1.6	9.38(0.76)	7.92(0.82)
C22:0	ND	0.5	ND	ND
C24:0	ND	0.5	ND	ND
Total SFA	27	30.6	28.97(1.08)	27.65(1.38)
C16:1n-7	7.3	38.89	11.02(0.78)	14.35(1.77)
C17:1	0.4	ND	ND	ND
C18:1n-9	14.4	0.7	6.36	8.12
C18:1n-9c	ND	ND	2.14(0.28)	1.49(0.23)
C18:1n9-t	ND	ND	4.22(1.37)	6.63(0.59)
C18:1n-7	2.5	1.1	ND	ND
C20:1	ND	ND	ND	1.19(0.63)
C22:1n-9	0.3	ND	ND	ND
Total MUFA	24.9	40.69	17.38(1.98)	23.66(2.18)
C16:2	ND	4.2	ND	ND
C16:3	ND	7.1	ND	ND
C16:4	ND	0.5	ND	ND
C18:2n-6	18.6	0.8	ND	0.59(0.35)
C18:3n-6	0.6	ND	ND	ND
C18:3n-3	11.3	0.45	ND	1.12(0.27)
C18:4n-3	6.8	0.5	ND	ND
C20:2	ND	ND	ND	0.77(0.17)
C20:3n-6	0.2	ND	ND	ND
C20:4n-6	0.2	4.8	2.19(0.18)	1.07(0.27)
C20:5n-3	0.5	9.1	28.74(0.91)	29.36(0.66)
C22:5n-6	ND	ND	ND	ND
C22:6n-3	9.9	1.3	23.58(3.21)	15.79(1.40)
Total PUFA	48.1	28.75	54.51(2.90)	48.69(0.89)

Supplementary Table 3.2. Fatty acid profiles of flesh samples from geoducks within 9 treatments and the reference group on day 36. Data are expressed as mean (±SD). ND denotes values not detected.

Fatty acid	Flesh day 36									
	L-L	L-M	L-H	M-L	M-M	M-H	H-L	H-M	H-H	Reference
C14:0	2.69(0.20)	3.26(0.54)	2.96(0.20)	1.70(1.52)	2.67(0.41)	2.90(0.25)	2.03(1.76)	3.20(0.26)	1.74(1.62)	3.20(0.25)
C15:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:0	16.03(0.67)	16.59(0.99)	15.68(0.40)	17.48(0.78)	17.6(0.28)	18.48(0.85)	16.85(0.35)	16.03(0.30)	17.53(0.63)	16.75(0.36)
C18:0	8.84(0.21)	9.39(0.24)	9.21(0.41)	10.10(1.02)	10.69(0.85)	9.77(0.29)	9.83(0.49)	10.44(0.77)	9.58(0.50)	8.68(0.77)
C22:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C24:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total SFA	27.56	29.24	27.85	29.28	30.96	31.15	28.71	29.67	28.85	28.63
C16:1n-7	11.19(0.29)	11.05(0.12)	10.43(0.30)	11.47(0.44)	11.01(0.64)	11.89(0.13)	11.87(0.64)	10.34(1.15)	11.49(0.44)	12.20(0.11)
C17:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:1n-9	6.37	6.92	6.76	8.04	8.55	8.82	7.01	7.81	7.58	6.86
C18:1n-9c	2.02(0.38)	2.66(0.26)	2.65(0.26)	2.60(0.33)	3.28(0.57)	3.14(0.31)	2.39(0.38)	3.41(0.70)	3.53(0.58)	2.26(0.89)
C18:1n9-t	4.35(0.77)	4.26(0.20)	4.11(0.44)	5.44(1.06)	5.27(0.40)	5.68(0.45)	4.62(0.50)	4.40(0.37)	4.05(1.59)	4.60(0.76)
C18:1n-7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C22:1n-9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total MUFA	17.56	17.97	17.19	19.51	19.56	20.71	18.88	18.15	19.07	19.06
C16:2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:2n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:3n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:3n-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:4n-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:3n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:4n-6	2.17(0.29)	2.61(0.22)	2.59(0.38)	2.30(0.41)	2.59(0.37)	2.17(0.20)	2.68(0.23)	2.95(0.29)	2.37(1.08)	1.46(0.42)
C20:5n-3	29.14(0.43)	26.66(1.62)	27.10(0.54)	26.43(1.40)	23.75(1.60)	25.21(1.23)	27.01(1.00)	29.48(1.24)	25.95(2.78)	30.16(1.71)
C22:5n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C22:6n-3	23.57(0.56)	23.52(0.38)	25.27(0.68)	22.48(2.31)	23.14(1.15)	20.75(1.00)	22.72(2.42)	24.24(1.91)	23.77(2.14)	20.69(0.89)
Total PUFA	54.88	52.79	54.96	51.21	49.48	48.13	52.41	56.67	52.09	52.31

Supplementary Table 3.3. Fatty acid profiles of flesh samples from geoducks within 9 treatments and the reference group on day 73. Data are expressed as mean (±SD). ND denotes values not detected.

Fatty acid	**Flesh day 73**									
	L-L	L-M	L-H	M-L	M-M	M-H	H-L	H-M	H-H	Reference
C14:0	1.25(1.28)	2.76(0.04)	2.50(0.78)	1.74(1.51)	1.44(1.41)	2.67(0.19)	1.37(1.30)	2.01(1.74)	3.33(0.22)	2.54(0.71)
C15:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:0	17.18(0.25)	18.18(1.35)	17.25(1.18)	17.02(0.78)	17.41(1.50)	17.56(1.29)	14.43(1.04)	15.52(0.92)	17.11(1.00)	16.22(0.27)
C18:0	10.94(0.22)	10.53(1.12)	10.43(0.64)	10.68(0.51)	10.89(1.55)	10.37(0.62)	12.05(1.15)	10.70(0.95)	10.22(0.36)	11.25(0.5)
C22:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C24:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total SFA	29.37	31.47	30.18	29.44	29.74	30.69	27.85	28.23	30.66	28.0
C16:1n-7	11.08(0.34)	11.39(0.30)	11.30(0.60)	11.34(0.19)	11.05(0.64)	11.31(0.70)	10.01(0.63)	10.69(0.39)	10.44(0.28)	9.30(0.5)
C17:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:1n-9	7.05	8.6	8.47	8.03	8.21	8.84	4.17	7.11	7.42	6.43
C18:1n-9c	3.09(0.54)	2.90(0.07)	3.16(0.47)	2.83(0.48)	3.24(0.36)	3.00(0.31)	2.25(1.99)	3.45(0.58)	3.55(0.20)	1.91(0.34)
C18:1n9-t	3.96(1.94)	5.70(0.58)	5.31(0.67)	5.20(0.59)	4.97(1.10)	5.84(0.41)	1.92(2.06)	3.66(1.04)	3.87(0.38)	4.52(0.03)
C18:1n-7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C22:1n-9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total MUFA	18.13	19.99	19.77	19.37	19.26	20.15	14.18	17.8	17.86	17.7
C16:2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:2n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:3n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:3n-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:4n-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:3n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:4n-6	1.31(1.26)	2.34(0.13)	2.90(0.33)	2.64(0.24)	2.16(0.50)	2.72(0.25)	4.51(1.03)	3.34(0.69)	3.40(0.28)	2.25(0.15)
C20:5n-3	25.85(0.99)	25.21(1.80)	24.96(1.73)	25.51(1.96)	24.93(2.25)	24.85(1.61)	24.33(2.10)	25.11(1.73)	23.65(1.42)	29.49(0.57)
C22:5n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C22:6n-3	25.34(2.77)	21.00(0.54)	22.20(1.43)	23.05(2.06)	23.90(3.10)	21.68(1.17)	29.13(4.37)	25.52(2.32)	24.43(1.07)	22.52(1.17)
Total PUFA	52.5	48.55	50.06	51.2	50.99	49.25	57.97	53.97	51.48	54.26

Supplementary Table 3.4. Fatty acid profiles of viscera samples from geoducks within 9 treatments and the reference group on day 36. Data are expressed as mean (±SD). ND denotes values not detected.

Fatty acid	**Viscera day 36**									
	L-L	L-M	L-H	M-L	M-M	M-H	H-L	H-M	H-H	Reference
C14:0	2.29(0.14)	2.84(0.76)	2.61(0.12)	2.58(0.14)	2.45(0.20)	2.73(0.24)	2.56(0.05)	2.77(0.36)	3.38(0.21)	2.87(0.33)
C15:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:0	16.14(0.09)	16.89(0.56)	16.14(0.75)	16.49(0.18)	16.08(0.24)	16.93(0.83)	16.66(0.36)	16.63(0.76)	17.40(0.60)	17.19(0.45)
C18:0	9.24(0.54)	8.96(0.79)	8.99(0.28)	9.31(0.63)	9.84(0.82)	8.68(0.28)	9.40(0.63)	10.04(0.55)	8.23(0.28)	8.58(0.76)
C22:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C24:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total SFA	27.67	28.69	27.74	28.38	28.37	28.34	28.62	29.44	29.01	28.64
C16:1n-7	9.85(0.17)	10.11(0.74)	16.14(0.18)	10.01(0.56)	9.91(0.79)	10.74(0.67)	10.96(0.20)	10.51(0.98)	12.67(2.26)	12.71(1.03)
C17:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:1n-9	9.54	7.92	8.02	7.25	7.32	8.07	6.87	7.96	7.85	8.36
C18:1n-9c	1.85(0.12)	2.27(0.36)	2.51(0.40)	1.74(0.15)	2.37(0.41)	2.41(0.24)	1.53(0.26)	2.31(0.10)	2.25(0.42)	1.52(0.17)
C18:1n9-t	7.69(1.66)	5.65(0.30)	5.51(0.25)	5.51(2.69)	4.95(1.21)	5.66(1.90)	5.34(1.46)	5.65(0.31)	5.60(0.11)	6.84(0.88)
C18:1n-7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:1	1.79(0.20)	1.88(0.24)	1.77(0.47)	1.87(0.36)	1.87(0.46)	1.90(0.07)	1.45(0.55)	2.20(0.32)	1.78(0.51)	1.79(0.18)
C22:1n-9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total MUFA	21.18	19.91	25.93	19.13	19.1	20.71	19.28	20.67	22.3	22.86
C16:2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:2n-6	0.50(0.36)	1.26(0.80)	1.35(0.34)	0.88(0.05)	1.10(0.39)	1.53(0.19)	0.83(0.26)	1.31(0.07)	1.36(0.50)	0.41(0.39)
C18:3n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:3n-3	1.22(0.13)	1.81(0.62)	1.85(0.08)	1.38(0.11)	1.66(0.20)	1.90(0.19)	1.34(0.14)	1.55(0.07)	1.64(0.48)	1.01(0.29)
C18:4n-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:2	0.60(0.41)	1.01(0.14)	1.02(0.32)	0.90(0.08)	1.10(0.13)	1.07(0.13)	0.95(0.11)	1.15(0.07)	1.04(0.15)	1.00(0.05)
C20:3n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:4n-6	1.84(0.14)	1.92(0.28)	2.14(0.29)	1.97(0.40)	2.51(0.54)	2.05(0.18)	2.16(0.31)	2.43(0.33)	1.84(0.49)	1.16(0.13)
C20:5n-3	28.50(1.32)	26.96(1.57)	26.75(1.82)	28.70(1.71)	26.18(2.39)	26.93(0.10)	28.74(0.17)	24.95(0.88)	26.14(2.19)	30.6(1.39)
C22:5n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C22:6n-3	18.48(0.69)	18.43(1.49)	19.34(1.61)	18.66(0.50)	19.95(1.27)	17.47(0.47)	18.09(1.11)	18.49(1.92)	16.69(0.65)	14.33(2.48)
Total PUFA	51.14	51.39	52.45	52.49	52.5	50.95	52.11	49.88	48.71	48.51

Supplementary Table 5. Fatty acid profiles of viscera samples from geoducks within 9 treatments and the reference group on day 73. Data are expressed as mean (±SD). ND denotes values not detected.

Fatty acid	**Viscera day 73**									
	L-L	L-M	L-H	M-L	M-M	M-H	H-L	H-M	H-H	Reference
C14:0	2.66(0.70)	2.51(0.03)	2.70(0.13)	2.82(0.20)	2.93(0.37)	2.61(0.18)	2.36(0.77)	2.92(0.14)	2.84(0.37)	3.28(0.32)
C15:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:0	16.94(0.28)	16.21(0.73)	16.16(0.81)	16.54(0.52)	16.40(1.05)	16.46(0.45)	16.18(1.00)	17.17(0.85)	17.35(0.43)	17.73(0.73)
C18:0	9.87(1.70)	9.52(0.40)	9.77(0.66)	9.17(0.23)	9.38(0.78)	9.27(0.46)	11.01(1.06)	9.93(0.49)	9.43(1.04)	8.64(0.11)
C22:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C24:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total SFA	29.47	28.24	28.63	28.53	28.71	28.34	29.55	30.02	29.62	29.65
C16:1n-7	10.00(1.12)	9.69(0.42)	9.90(0.70)	10.22(0.48)	10.42(0.54)	10.65(0.42)	10.08(1.35)	10.68(0.31)	10.54(0.90)	13.19(1.30)
C17:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:1n-9	8.21	8.23	6.88	9.98	8.62	8.38	8.04	8.04	7.89	7.92
C18:1n-9c	2.30(0.16)	2.53(0.18)	2.79(0.24)	2.16(0.26)	2.31(0.35)	2.22(0.06)	2.45(0.08)	2.47(0.22)	2.45(0.15)	1.32(0.14)
C18:1n9-t	5.91(3.38)	5.70(0.87)	4.09(1.88)	7.82(1.04)	6.31(0.45)	6.16(2.54)	5.59(1.00)	5.57(0.17)	5.44(0.72)	6.60(1.08)
C18:1n-7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:1	0.97(1.02)	1.56(0.47)	2.03(0.33)	1.52(0.44)	2.04(0.17)	1.68(0.46)	0.89(0.77)	1.59(0.77)	1.83(0.29)	1.47(0.28)
C22:1n-9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total MUFA	19.18	19.48	18.81	21.72	21.08	20.71	19.01	20.31	20.26	22.58
C16:2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:2n-6	0.72(0.63)	1.02(0.31)	1.53(0.36)	0.91(0.35)	1.31(0.27)	1.25(0.30)	0.69(0.60)	1.23(0.15)	1.42(0.21)	0.55(0.18)
C18:3n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:3n-3	1.36(0.73)	1.65(0.25)	2.14(0.42)	1.68(0.11)	1.86(0.07)	1.79(0.19)	1.42(0.23)	1.76(0.26)	1.71(0.29)	1.08(0.14)
C18:4n-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:2	0.44(0.46)	1.56(0.47)	0.85(0.21)	0.59(0.51)	0.97(0.08)	0.92(0.24)	0.67(0.59)	0.96(0.08)	1.05(0.09)	0.89(0.08)
C20:3n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:4n-6	2.00(0.24)	2.33(0.12)	2.89(0.69)	1.93(0.19)	2.09(0.36)	2.20(0.21)	2.55(0.61)	2.07(0.40)	2.15(0.45)	1.26(0.06)
C20:5n-3	26.83(2.00)	27.22(0.47)	25.79(0.95)	26.92(1.26)	26.14(0.50)	26.97(1.70)	26.25(1.19)	25.55(0.83)	25.52(1.29)	29.49(1.82)
C22:5n-6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C22:6n-3	19.98(2.16)	19.60(0.97)	19.37(1.02)	17.71(1.24)	17.83(1.16)	17.81(0.78)	19.86(3.71)	18.11(0.36)	18.28(1.09)	14.50(0.72)
Total PUFA	51.33	53.38	52.57	49.74	50.2	50.94	51.44	49.68	50.13	47.77

CHAPTER 4 – Practical fertilization procedure and embryonic development of the New Zealand geoduck clam (*Panopea zelandica*)

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Abstract

Despite the fact that a successful aquaculture industry for the geoduck *Panopea zelandica* (Quoy and Gaimard, 1835) must rely on hatchery spat production, little is known about the embryonic development, which appears to be critical for larval success in this species. The present study investigated the development of *P. zelandica* embryos at 15°C and 35 ppt and the optimal sperm:egg ratios for fertilization under hatchery conditions. *P. zelandica* broodstock were induced to spawn by serotonin injection. Sperm and eggs were collected within 30 min post-release, and then stored at 4°C for up to 4 h and 1.5 h, respectively. Fertilization was conducted at sperm:egg ratios of: 50:1, 100:1, 500:1, 1000:1, and 10,000:1 with a sperm-egg contact time of 40 min. The optimal sperm:egg ratio was determined to be < 500:1 and the normal embryo yield at 3 and 18 h post-fertilization ranged from 83-96%. *P. zelandica* eggs (~ 80 µm diameter) developed the first and second polar bodies within 15 - 20 and 50 - 55 min post-fertilization, respectively. The blastula appeared at ~ 8 h post fertilization (hpf), including the X^R and X^L cells and the presumptive shell field depression. Gastrulation occurred at 12 - 18 hpf with organic material shell apparent at the shell field depression. The mid-stage trochophore, which appeared at around 35 hpf had an apical plate with an apical tuft. The shell field spread to form the periostracum, which expanded and folded into right and left segments covering the late trochophore. The early D-stage veliger appeared at 45 hpf with the soft body being enclosed by two valves and the appearance of the velum. These observations will serve as the basis for future analyses of *P. zelandica* embryogenesis and for optimization of commercial production of D-veliger larvae.

4.1 Introduction

The New Zealand aquaculture sector has set a target to achieve annual sales of $1 billion NZD by 2025 (Carter, 2012), more than doubling current revenues. Alongside adding value to existing aquaculture species (e.g. salmon, Pacific oysters, Greenshell™ mussels), another strategic priority to accomplish this goal is to identify new shellfish species with commercial potential and develop techniques for their production (Carter, 2012). Geoduck are a high value species, currently selling for up to $200 - $300 USD/kg in Asian restaurants (Shamshak and King, 2015). The endemic geoduck clam *Panopea zelandica* (Quoy and Gaimard, 1835) has been chosen as an emerging species for aquaculture within this strategy (King, 2010). *P. zelandica* populations have been found in both North and South islands of New Zealand (Breen et al., 1991; Gribben et al., 2004). However, the wild fishery is unlikely to sustainably fulfil potential market demands (see review in Gribben and Heasman, 2015). Thus, geoducks have become an object of significant aquaculture research and development.

The success of any shellfish aquaculture depends on the availability of seed/spat to stock farms. For many bivalves, such as mussels and oysters, intensive recruitment of wild juveniles onto spat-catching ropes or frames can result in a relatively efficient way to obtain wild seed to supply the farms (Buestel et al., 2009; Alfaro et al., 2010). However, geoduck spat do not attach or cement to substrates but bury in sand. This attribute makes it practically impossible to collect wild geoduck spat; hence, the geoduck aquaculture industry must rely on hatchery-based spat production.

Successful embryo development is critical for reliable spat production. The yield of embryos can be substantially affected by the ratio of sperm:egg during fertilization (Dong et al., 2012). For example, low sperm:egg ratios can reduce the probability of gamete contact, while high ratios can increase the risk of polyspermy (Gribben et al., 2014). Polyspermy can then cause dissolution of egg membranes and abnormal embryo development (Stephano and Gould, 1988; Clotteau and Dubé, 1993; Encena et al., 1998). Abnormal embryos either terminate prior to the shell development or result in deformed D-larvae, which cannot survive to the pediveliger stage. Hence, it is important to determine the optimal sperm:egg ratio so that polyspermy can be avoided without compromising fertilization rates. This optimal ratio varies among different bivalve species. For example, a sperm:egg ratio of 10,000:1 is optimal for the cockle *Clinocardiuim nuttallii* (Liu et al., 2008), whereas 1000:1 is optimal for the oysters *Crassostrea virginica* and *Crassostrea gigas* (Alliegro and Wright, 1985; Stephano and Gould, 1988), and a ratio of ≤ 200:1 is ideal for the blood clam *Tegillarca granosa* (Dong et al., 2012).

During experimental spat production runs at a research hatchery (Cawthron Institute, Nelson, New Zealand), a high number of deformed geoduck D-larvae were observed, resulting in very low larval survival by day seven post-fertilization. Polyspermy was implicated in the deformities as the high number of sperm was observed surrounding eggs at fertilization phase in those low spat production trials. Deformities are also often associated with high vulnerability to pathogenic bacterial infection, which can be contagious to healthy larvae within the same batch (Elston, 1993). Although the hatchery production of Pacific geoduck (*Panopea generosa*) spat is commercially well-established in the USA and Canada, limited information

on optimal sperm:egg ratios has been released. However, in a study to investigate the production of triploid *P. generosa*, Vadopalas and Davis (2004) successfully used a sperm:egg ratio of 40:1. More recently, in New Zealand, Gribben et al. (2014) conducted a comprehensive study to investigate the fertilization kinetics of *P. zelandica*, and recommended a broad sperm:egg ratio of 5,000–50,000:1 for hatchery production with fresh gametes (< 30 min old), a starting egg density of 20 eggs mL^{-1}, and a sperm-egg contact time of 5 – 10 min. Under these conditions, greater sperm densities resulted in high percentages of polyspermy and poor fertilization success. While the fertilization kinetics model provided highly valuable information, the suggested gamete age and sperm-egg contact time by Gribben et al. (2014) might not be feasible for hatchery operators to conduct commercial-scale fertilization. It is well-established that gamete age and sperm-egg contact time considerably affects fertilization success and the optimal sperm:egg ratio (Levitan, 2006; Stephano and Gould, 1988). A more practical commercial scenario would be to cold-store gametes for up to 2 h, enabling a sufficient number of eggs to be used (Adams et al., 2004), and then to provide sperm-egg contact times of > 30 min in order to evaluate fertilization success as is routine with other bivalve species (Helm et al., 2004). Thus, there is a need to determine the optimal *P. zelandica* sperm:egg ratio for commercial fertilization purposes.

Fundamental biological knowledge of embryonic and larval development is very important for the hatchery culture of bivalves. Bivalve embryogenesis has two notable features that relate to organ development and shell formation of early larvae (Kin et al., 2009). The cleavage pattern feature determines the normal development of embryos, and consequently the normal development of organs,

such as the velum, mouth, apical tuft and stomach in D-larvae (Hashimoto et al., 2014). The normal shape and integrity of larval shells is dependent on the successful cleavage and development of the shell-founding cell during the zygote and morula stages, the invagination, evagination, and expansion of the shell field during the gastrula stage, and the secretion of shell matrices and calcification during the trochophore stage (Kin et al., 2009). Surprisingly, there are very few studies on embryonic development for any geoduck species. Most studies on geoduck embryos have only focused on the effects of temperature and salinity on the success of embryogenesis (i.e., D-larval yield), but not on the embryonic development itself. A detailed description of geoduck embryogenesis, particularly the timing of developmental stages and characterization of key phenotypes, would be extremely valuable for future advancements and optimization of hatchery technologies. Specifically, without information on optimal sperm:egg ratios and embryonic development, the deformities we have observed in geoduck larvae may not be well understood and the yield of larvae and spat may not be reliably optimized. Thus, the aims of the current study are to determine the optimal sperm:egg ratio under hatchery conditions and describe the normal embryonic development in *P. zelandica*.

4.2 Materials and methods

4.2.1 Broodstock conditioning and gamete collection

P. zelandica broodstock (105 – 130 mm shell length, 500 - 800 g live weight) were collected from Golden Bay (South Island, New Zealand) and conditioned in flow-through 1 µm-filtered seawater at 15°C with microalgae (*Tisochrysis lutea* and *Chaetoceros muelleri*, 1:1 cell counts) for 3 months (after Le et al., 2014).

Geoduck broodstock were induced to spawn by injecting 1 – 2 mL of 2 mM serotonin solution into their mantle. After their sex was revealed by initial gamete release, males and females were separated into different containers. Gametes were collected within 30 min of release, then rinsed through a 100 µm sieve to remove particulate matter. Eggs were caught on a 40 µm mesh screen and re-suspended in 500 mL seawater. Sperm and egg solutions were then stored at 4°C for up to 2 h and 1 h, respectively. Before fertilization, gametes were examined for quality and quantity. All gametes were in good quality according to the characterization of egg shape and sperm motility as in Baker and Tyler (2001). Sperm and egg concentrations were determined from three replicate counts of 20 µL and 200 µL aliquots, respectively. Sperm aliquots were diluted 1000x, transferred to a haemocytometer, and cells were counted under a light microscope (Olympus, BX41) at 400x magnification. Egg densities were counted under 200x magnification under an inverted light microscope (Olympus, CKX41).

4.2.2 Sperm:egg ratio optimization trial

Approximately 3000 eggs from one female were fertilized and incubated at 15°C in each of fifteen 50 mL Falcon™ tubes containing 30 mL of 1 µm filtered seawater and 4 µmol EDTA. Sperm aliquots from one male were pipetted into the Falcon tubes to provide sperm:egg ratios of 50:1, 100:1, 500:1, 1000:1, and 10,000:1 (3 replicates for each ratio). After a 40 min contact time, embryos and any unfertilized eggs were filtered and washed on a 22 µm mesh screen to remove excess sperm. Samples were incubated in 50 mL Falcon™ tubes containing fresh 1 µm filtered seawater with 4 µmol EDTA. After 3 and 18 h post fertilization (hpf), embryos were carefully resuspended and 1 mL of each embryo suspension was fixed in

Davidson's solution and stored at 4°C for subsequent visual assessment. A total of 2230 and 3890 embryos were assessed in the 3 and 18 hpf groups, respectively. The embryonic development was assessed visually at 400x magnification using a light microscope (Olympus, CKX41). Embryos that showed signs of irregular cleavage, incomplete blastula development and discoloration were recorded as 'abnormal' (Lewis and Galloway, 2009). Unfertilized eggs were also categorized as 'abnormal' for ease of calculation. The proportion of normally-developed embryos was determined by expressing the number of normal embryos as a percentage of the number of eggs initially present.

4.2.3 Embryonic development

Approximate 1 million eggs were fertilized in a 10 L bucket with a sperm:egg ratio of 500:1, screened (22 µm) and washed with fresh 1 µm-filtered seawater. Approximately 500,000 embryos were transferred to a beaker containing 5 L of 1 µm-filtered seawater and 4 µmol EDTA. The temperature of the incubation seawater was maintained at 15°C in a thermostat-controlled incubator. Triplicate 1 mL samples of suspended embryos were pipetted from the 5 L beaker every 10 min for the first 2 h, then every 30 min for the next 4 h, and every 2 h thereafter until the D-veliger larval stage. Samples were fixed in Davidson's solution and stored at 4°C until visual assessment. Embryos were observed using a light microscope and a scanning electron microscope (SEM, Hitachi SU-70 Skottky). The cleavage pattern was described following the standard terms in Hashimoto et al. (2015).

4.2.4 Scanning electron microscopy

Preserved embryos were washed with phosphate buffer (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH = 7.4) for 5 min, then rinsed for 1 min with deionized water. Embryos were then dehydrated through an ascending series of analytical grade ethanol 50, 60, 70, 80, 90, and 100% for 15 min intervals each (Turner and Boyle, 1974). After dehydration, samples were soaked in 98% chloroform for 30 sec, and then dried for 12 h in a desiccator. To dry samples in a vaporous condition, a chloroform-soaked filter paper was also placed in the desiccator as suggested by Wassnig and Southgate (2012). Dried samples were placed on adhesive carbon discs and mounted on aluminium stubs. Samples were sputter coated with carbon for 40 sec using an ion sputter coater (Hitachi E-1045), then imaged via SEM at 5.0 kV.

4.2.5 Statistical analysis

The percentages of normal embryos were arcsine-transformed (Sokal and Rohlf, 1995) to achieve homogeneity of variance and normality. The effect of sperm:egg ratios on the normal embryo yield were analyzed by one-way analysis of variance (ANOVA), followed by Tukey pairwise comparison at the 95% significance level using the statistical software Minitab v. 17. All data are expressed as mean ± SD.

4.3 Results

4.3.1 Sperm:egg ratio

The sperm:egg ratio significantly affected the percentage of normally-developed embryos at 3 hpf (df = 14, F = 6.62, $P = 0.007$) and at 18 hpf (df = 14, F = 27.99, $P < 0.001$; Fig. 4.3). The highest normal embryo percentage was achieved at a sperm:egg ratio of 50:1 as confirmed by both the 3 hpf and 18 hpf sampling events.

The percent of normal embryos after 3 hpf with a sperm:egg ratio of 50:1 was significantly higher than those obtained from 500:1 and 10,000:1 ratios. However, there was no significant difference in the quantity of normal embryos obtained after 3 hpf between sperm:egg ratios of 50:1 and 100:1. Moreover, at 18 hpf, significantly higher numbers of normal embryos were obtained at a sperm:egg ratio of 50:1 compared with those obtained at ratios of 1000:1 and 10,000:1 (Fig. 4.3). However, there was no significant difference in normal embryo development after 18 hpf among sperm:egg ratios from 50:1 to 500:1. Overall, there was a decreasing trend in the quantities of normally-developed embryos as the sperm:egg ratio increased.

4.3.2 Embryo development

Newly released eggs were pear-shaped and then became more spherical (with a diameter of 75 - 80 µm) immediately post-spawning. The first polar body became evident after 15 – 20 min post-fertilization (Fig. 4.1a, 4.2a). The second polar body was typically observed about 35 min later (50 – 55 min post-fertilization; Fig. 4.1b). The first cleavage started with polar lobe formation occurring at 1.5 hpf from the vegetal region, resulting in two unequal cells (small cell: AB, and large cell: CD; Fig. 4.1c, 4.2b). The polar body was located in the plane of cleavage. The second cleavage appeared at 2.5 hpf. Polar lobe formation occurred again, producing three smaller cells of similar size, referred to as the A, B, and C blastomeres and one larger blastomere (D; Fig. 4.1d, 4.2d). The third cleavage occurred at 4 hpf. The third cleavage was uneven, creating the first quartet of smaller apical micromeres (1a – 1d; Fig. 4.1e, 4.2e). The fourth cleavage occurred at 5 hpf, producing 16-cell embryos with the second micromere quartet ($1a^2$ – $1d^2$; Fig. 4.2f).

The fifth cleavage appeared at 6 hpf, producing 32 - cell embryos, or morulae, with the third micromere quartet (Fig. 4.1f, 4.2g - h).

The blastula appeared at ~ 8 hpf and showed a symmetric division pattern. The bilaterally-symmetric cell division yielded X^L and X^R regions and a presumptive shell field (Fig. 4.1h - i, 4.2i - l). Occasional cilia were apparent surrounding the anterior circular margin, forming the early prototroch. Two cellular depressions started at the late blastula within the vegetal side. The shell field depression in the dorsal region was recognizable as a crescent-shaped orifice in the blastomere X region. The other depression within the ventral region represented the blastopore. The early gastrula appeared at 12 hpf. The shell field and blastopore depressions at this stage were deeper than at the blastula stage (Fig. 4.2m - n). The prototrochal pad developed and correlated well with the general timing at which embryos began rotating, following circular trajectories within the water column. The gastrulation appeared at 18 hpf, by which time overall shape was no longer spherical. The dorsal region was distinguishable by an open orifice, which expanded under and posterior to the developing prototrochal pad (Fig. 4.2o). The new shell material (pellicle) appeared as a wrinkle and accumulated at either side of the orifice. A mid-stage trochophore appeared at around 35 hpf. The trochophores were ovoid with a broad animal region and narrower vegetal region (Fig. 4.2p). The well-developed prototroch was characterized as a crown of motile cilia (Fig. 4.1j) and divided the trochophore into two regions (Fig. 4.2p). The posterior region contained the blastopore on the ventral side and the shell field on the dorsal side. The anterior region contained the apical plate on which the cilia elongated and thickened to form an apical tuft, which acts as a sensory organ (Fig.

4.2p - r). The cilia developed on the posterior area of embryos and formed the presumptive telotroch (Fig. 4.2s). Late-stage trochophores appeared at 39 hpf. The shell field spread out to form a flat and smooth periostracum on the posterior-dorsal region (Fig. 4.2t). The periostracum then expanded and folded into right and left segments covering the trochophore (Fig. 4.2u). Early D-stage veligers appeared at 45 hpf with the soft body enclosed by two valves and the appearance of the velum (Fig. 4.2v - x). The mineralization began along the hinge, and then continued along the shell edge while the center of the valve remained uncalcified. A summary of the timing of development stages is given in Table 4.1.

4.4 Discussion

4.4.1 Sperm:egg ratio

The reported values of optimal sperm:egg ratios for fertilization and successful development vary greatly for different bivalve species. The low range of sperm:egg ratio (≤ 100:1), which was found to be optimal for normal embryo yields for the New Zealand geoduck *P. zelandica* in the present study was also used for the Pacific geoduck *P. generosa* by Vadopalas and Davis (2004). In contrast, Gribben et al. (2014) found the ultra-high range (≥ 10,000:1) of sperm:egg ratio to be optimal for fertilization of the *P. zelandica*. The low range of sperm:egg ratio (≤ 100:1) has also been found to be optimal for fertilization in the scallop *Placopecten magellanicus* (Desrosiers et al., 1995) and the clams *Spisula solidissima* (Clotteau and Dubé, 1993), and *Tegillarca granosa* (Dong et al., 2012). Meanwhile, the medium range of sperm:egg ratio (100-1000:1) has been found to optimize D-veliger larval yields in *C. gigas* (Song et al., 2009) and normal embryo yields in *C. gigas* (Stephano and Gould, 1988) and *C. virginica* (Alliegro and Wright, 1985). In

addition, a high range of sperm:egg ratio (1000-5000:1) has been found to be optimal for normal D-larvae yield in the oyster *Crassostrea rhizophorae* (Santos and Nascimento, 1985) and the scallop *Chlamys asperrima* (O'Connor and Heasman, 1995). An even higher range of sperm:egg ratio (≥ 10,000:1) has been found to be optimal for fertilization in the cockle *Clinocardium nuttallii* (Liu et al., 2008).

The fertilization rate for *P. zelandica* (81 – 91% 3 hpf and 88 – 96% 18 hpf) in the present study is higher than that (max. 70% 9 hpf) reported by Gribben et al. (2014). The procedures common to both the present study and Gribben et al. (2014) are spawning method and sperm motility evaluation before fertilization. Hence, besides sperm:egg ratios, other factors which potentially contributed to the difference in fertilization rate between these two studies might be egg density, oocyte maturation or/and contact time. The egg density was fixed at 100 mL^{-1} in the present study, while 20 eggs mL^{-1} were used by Gribben et al. (2014). Clotteau and Dubé (1993) have illustrated the importance of egg density in fertilization with the clam *S. solidissima*, reflected by the fertilization rate of the scallop *C. asperrima*, which significantly increased at higher egg densities of ≥ 500 eggs mL^{-1} (O'Connor and Heasman, 1995). In contrast, Levitan et al. (1991) did not find an effect of egg concentration on fertilization for the sea urchin *Strongylocentrotus franciscanus*; however their lowest egg concentration was over 600 eggs mL^{-1}. Hence, further investigation of the effect of egg density on the fertilization rate for *P. zelandica* should be conducted. The sperm-egg contact time in the present study (40 min) was also longer than that (5 - 10 min) used by Gribben et al. (2014). Interestingly, Gribben et al. (2014) also observed fertilization

at low sperm concentrations if the contact time was increased. Another potential factor influencing the higher normal embryo yield or lower polyspermy in the present study may be the age of eggs prior to fertilization (1.5 h), which was a longer storage period than that (< 30 min) used by Gribben et al. (2014). It must be noted that *P. zelandica* eggs obtained in the present study and Gribben et al. (2014) were the result of serotonin-induced spawning. Serotonin-spawned eggs have been suggested to be more vulnerable to polyspermy (Misamore et al. 1996). However, the polyspermic susceptibility of serotonin-spawned eggs can be reduced if incubated for over 1 h (O'Connor and Heasman, 1995). For example, the incidence of polyspermy of *C. gigas* artificially stripped eggs was significantly reduced from 98 to 4% if eggs were incubated 1 - 1.5 h prior to fertilization (Stephano and Gould, 1988).

In addition to those potential factors mentioned above (i.e. egg density, contact time and egg age), the temperature for storing gametes is a critical factor influencing fertilization practices and success. Gribben et al. (2014) found that *P. zelandica* gametes stored at 15°C for over 30 min had reduced viability. This reduction in viability has also been observed in other bivalves (e.g. *Clinocardium nuttallii*, Liu et al., 2008) at their spawning temperatures. However, when gametes are stored at lower temperatures the gamete viability can be maintained for up to 1.5 - 4 h (O'Connor and Heasman, 1995; Liu et al., 2008; Adams et al., 2004, 2009). Similarly, in the present study no negative effect of storing *P. zelandica* gametes at 4°C was found. Thus, it seems that reducing the temperature may be a factor in resolving inconsistencies between the age of eggs and their susceptibility to polyspermy.

Inevitably, in a commercial operation, eggs need to be pooled until sufficient quantities have been collected to stock an incubation tank, which may take several hours. Thus, cold storage adds flexibility to spawning and fertilization times and prolongs the viability of both sperm and egg. Further research might usefully be focused upon the mechanisms underlying the viability of geoduck gametes at low temperatures.

4.4.2 Embryonic development

The developmental time of *P. zelandica* embryos to D-veliger larvae in the present study was < 65 h at 15°C and < 48 h at 17°C in our commercial batches (unpublished data). These developmental periods were similar to those determined for *P. japonica* by Lee and Rho (1997), who incubated embryos at 14 and 17°C (Table 4.1). However, the incubation period for *P. japonica* embryos could be shortened to 27 h at 19°C (Nam et al., 2014). While it may be beneficial for geoduck hatcheries to maximize the developmental rate, the thermal threshold for normal development should not be exceeded (Santo and Nascimento, 1985). Thus, the development of *P. zelandica* embryos at higher temperatures may be examined in future research, to improve hatchery efficiency and understanding impacts of climate change.

In the current study, the formation times for the first and second polar bodies at 15°C and 35 ppt were 20 - 25 and 50 - 55 min, respectively. The appearance times of the second polar body of *P. zelandica* observed in this study were similar to those of the geoduck *P. generosa* at 15°C (Vadopalas and Davis, 2004). This information is important for the triploidy induction in bivalves, when using chemicals

to block the second polar body formation (Barber et al., 1992; Gerard et al., 1994; Vadopalas and Davis, 2004).

The present study provides the first record of early shell formation in geoducks. The presumptive shell field depression appeared at the blastomere X of *P. zelandica* blastula and started to depress at late blastula stage. The shell field depression occurring when the X^R and X^L were still present may confirm that the differentiation of the shell gland in *P. zelandica* occurs at the late blastula stage, while there are only a small number of cilia associated with the prototroch, and the embryos are spherical. The commencement of shell field depression in *P. zelandica* embryos was earlier than in other clams, e.g. *Ruditapes decussatus* (gastrula stage, Aranda-Burgos et al., 2014) and *Spisula solidissima* (early trochophore stage, Eyster and Morse, 1984). The process of shell field depression at the gastrula stage for *P. zelandica* was similar to that of other clams (e.g. *Chione cancellata*, *Venerupis pullastra*, and *Ruditapes decussatus*) in which the shell field did not undergo invagination (Mouëza et al., 2006; Aranda-Burgos et al., 2014). However, the shell invagination needed to close either completely or partially before the shell could be formed in other bivalves (e.g. the mussel *Mytilus galloprovincialis* (Kniprath, 1980), the scallop *Pecten maximus* (Casse et al., 1998), the clam *Spisula solidissima* (Eyster and Morse, 1984), and the oysters *Saccostrea kegaki* (Kin et al., 2009) and *C. gigas* (Zhang et al., 2012). This study also revealed that the shell mineralization only commenced once the periostracum covered the whole embryo, and began along the hinge, then continued along the shell margin, but did not initially include the center of the valves. This shell mineralization process was similar to *M. galloprovincialis* (Kniprath, 1980) and

Tridacna squamosa (LaBarbera, 1974). Furthermore, we observed that the shell valves preceded the ligament formation in *P. zelandica*. The same observation has been reported in *C. cancellata* (Mouëza et al., 2006).

In conclusion, sperm:egg ratios of 50-500:1 with a 40 min sperm-egg contact time gave the highest normal embryo yield under hatchery conditions. Eggs and sperm can be stored at 4°C to extend their viability up to 1.5 h, making the fertilization practical since geoducks typically continue to spawn for 4 h. In addition, incubating eggs at 4°C for over 1 h may make the eggs less susceptible to polyspermy. Embryo cleavage follows a spiral and unequal pattern while the shell field depresses and expands to create the periostracum. However, the ligament is not formed until the shell field covers the entire embryo. Further research is needed to determine the extent to which cold storage can prolong gamete viability, and whether incubation times exceeding 1 h can reduce the polyspermic susceptibility of eggs, as well as confirming the shell field pattern for *P. zelandica*.

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Table 4.1 The approximate post-fertilization developmental time sequence for geoduck embryos. *P. zelandica* data are derived from the current study and compared to *P. japonica* raised at different temperatures by Lee and Rho (1997).

Stage	***P. zelandica* at 15°C**	***P. japonica* at**		
		11 °C	**14 °C**	**17 °C**
1st polar body	15 - 20 min			
2nd polar body	50 - 55 min			
2 cells	1.5 h	2 h		
4 cells	2.5 h	4 h		
8 cells	4 h	9 h	5.4 h	4.3 h
16 cells	5 h	15 h		
32 cells	6 h			
Morula	6 h			
Blastula	8 h	23 h	18.7 h	12.3 h
Early gastrula	12 h			
Gastrula	18 h			
Early trochophore	28 h			
Trochophore	35 h	2 d	33.8 h	23.6 h
Late trochophore	39 h			
Early veliger	45 h			
D-Veliger	62 h	3 d	62.4 h	42.7 h

Table 4.2 List of abbreviations

ap	apical plate
at	apical tuft
b	blastopore
Ci	cilia
dp	depression
h	hinge
pb	polar body
pel	pellicle
ps	periostracum
psb	pseudo-blastopore
pSF	presumptive shell field
pt	prototroch
s	shell
SF	shell field
sp	sperm
tt	telotroch
Ve	velum

Figure 4.1 Light microscopy images of P. zelandica embryonic development. a) - e) initial cell divisions; f) morula; g) blastula; h) - i) gastrula and j) trochophore. Abbreviations are summarized in Table 4.2

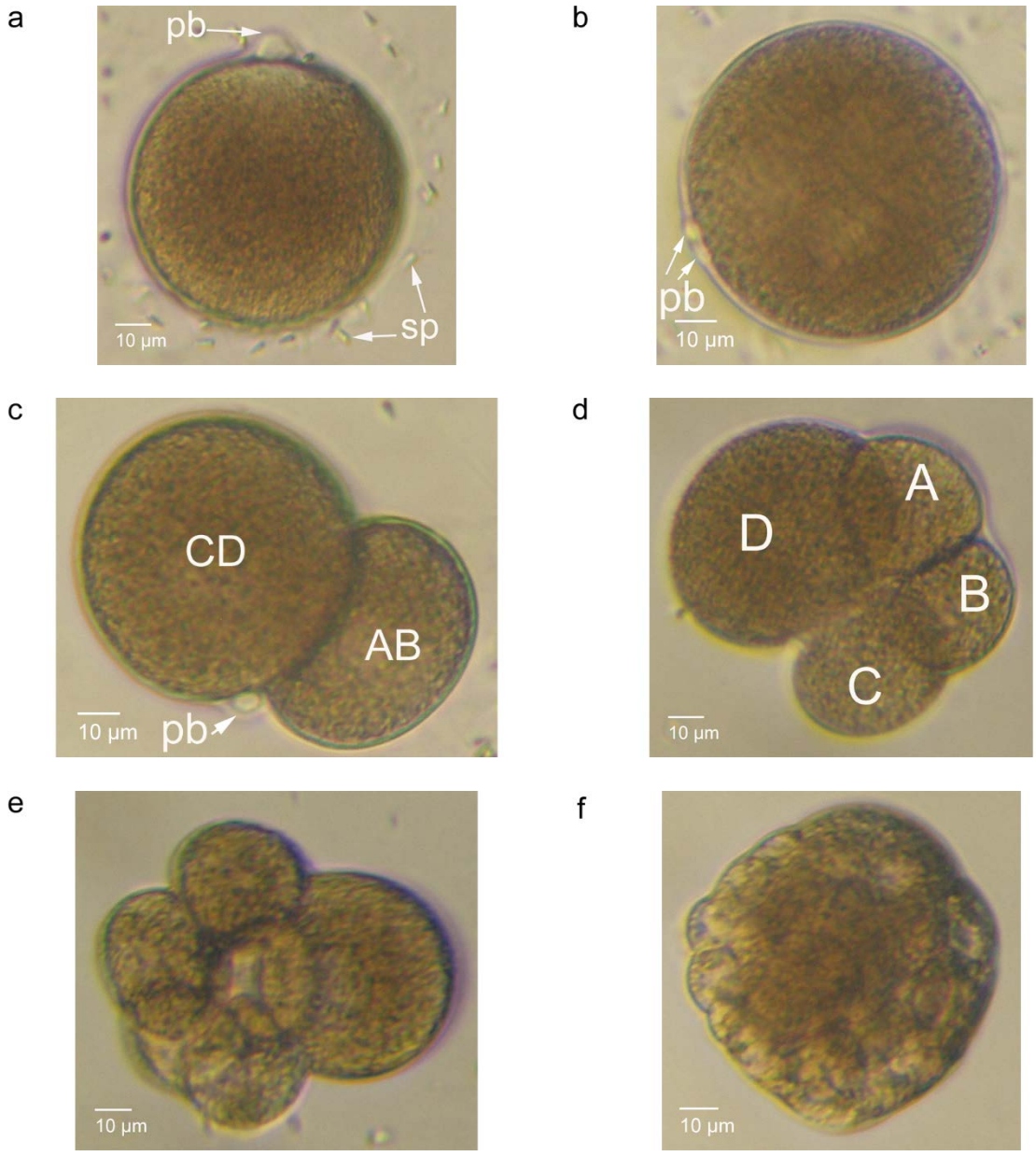
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Figure 4.1 cont.

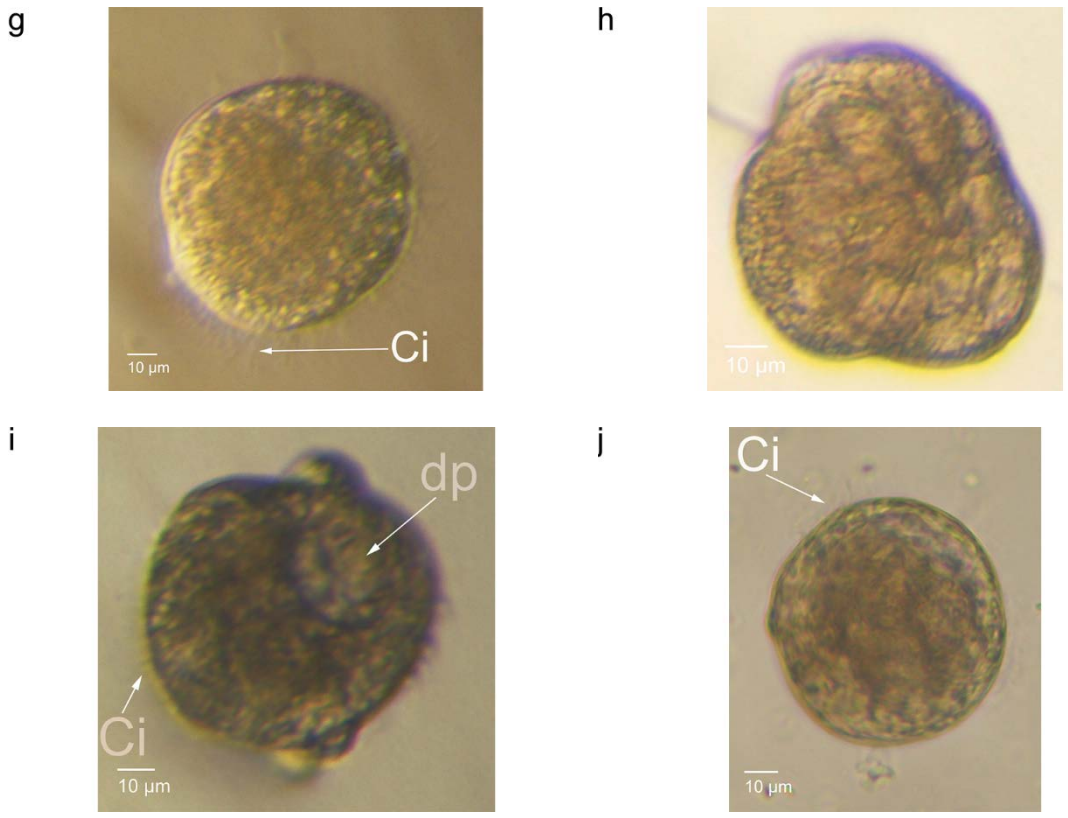
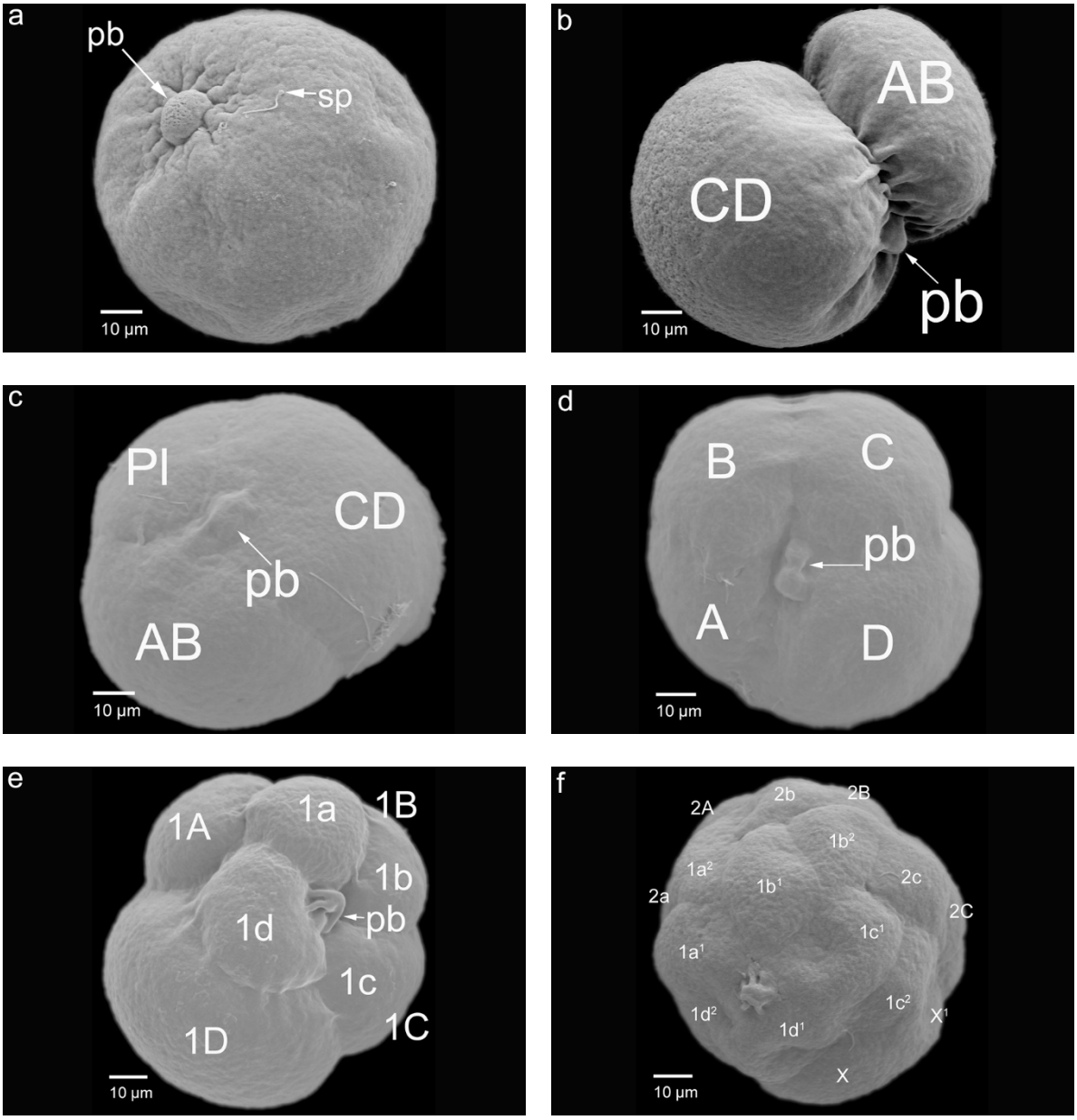


Figure 4.2 SEM images of *P. zelandica* embryonic development. a) fertilized egg; b) 2 cell stage; c) 3 cell; d) 4 cell; e) 8 cell; f) 16 cell; g) 32 cell; h) morula; i) - j) early blastula; k) - l) late blastula; m) - n) early gastrula; o) gastrula; p) - s) trochophore; t) - v) late trochophore and w) - x) early D-veliger. Abbreviations summarized in Table 4.2 and the results text.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f
2A
2b
2B
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1a²
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Figure 4.2 cont.

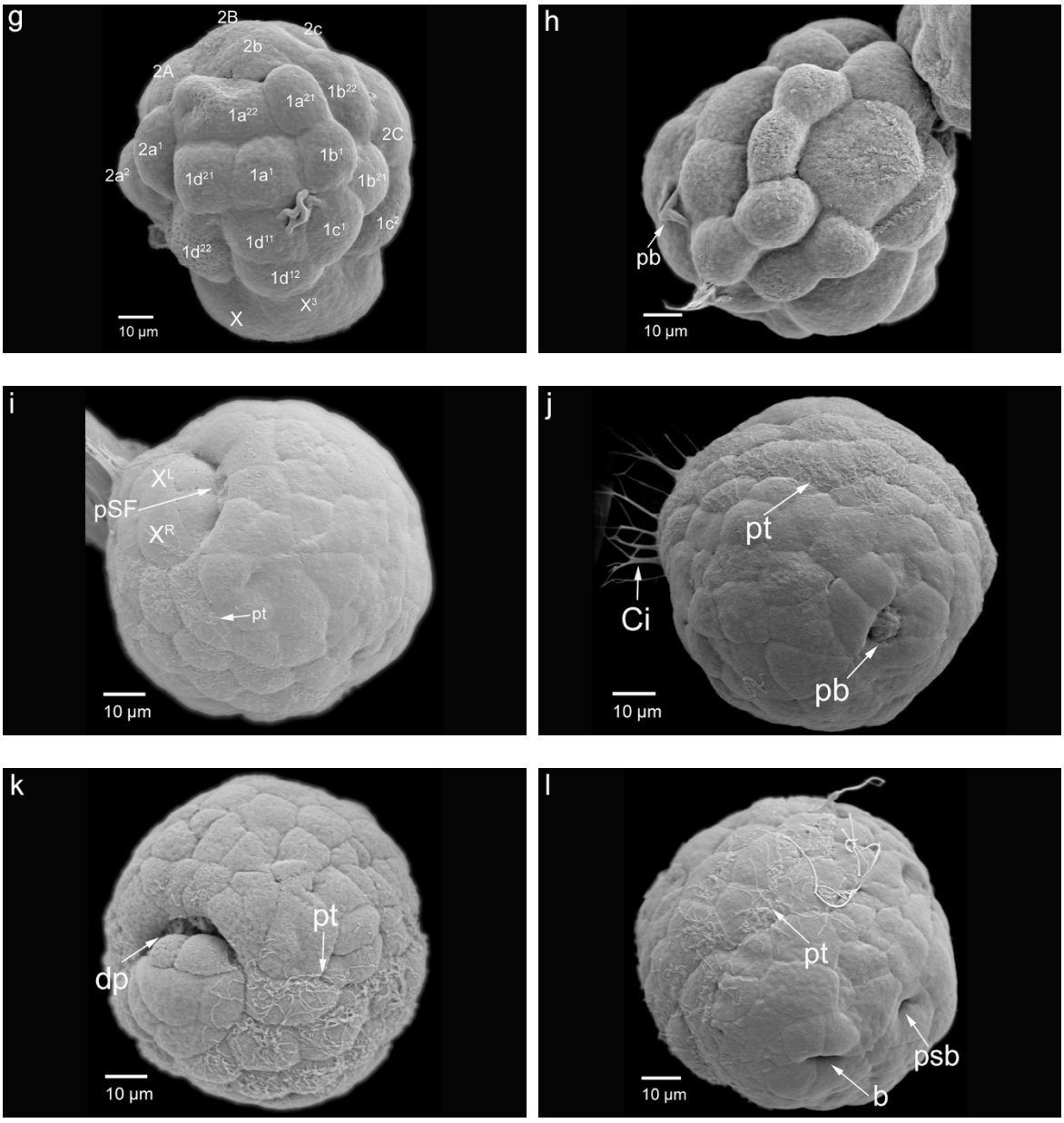


Figure 4.2 cont.

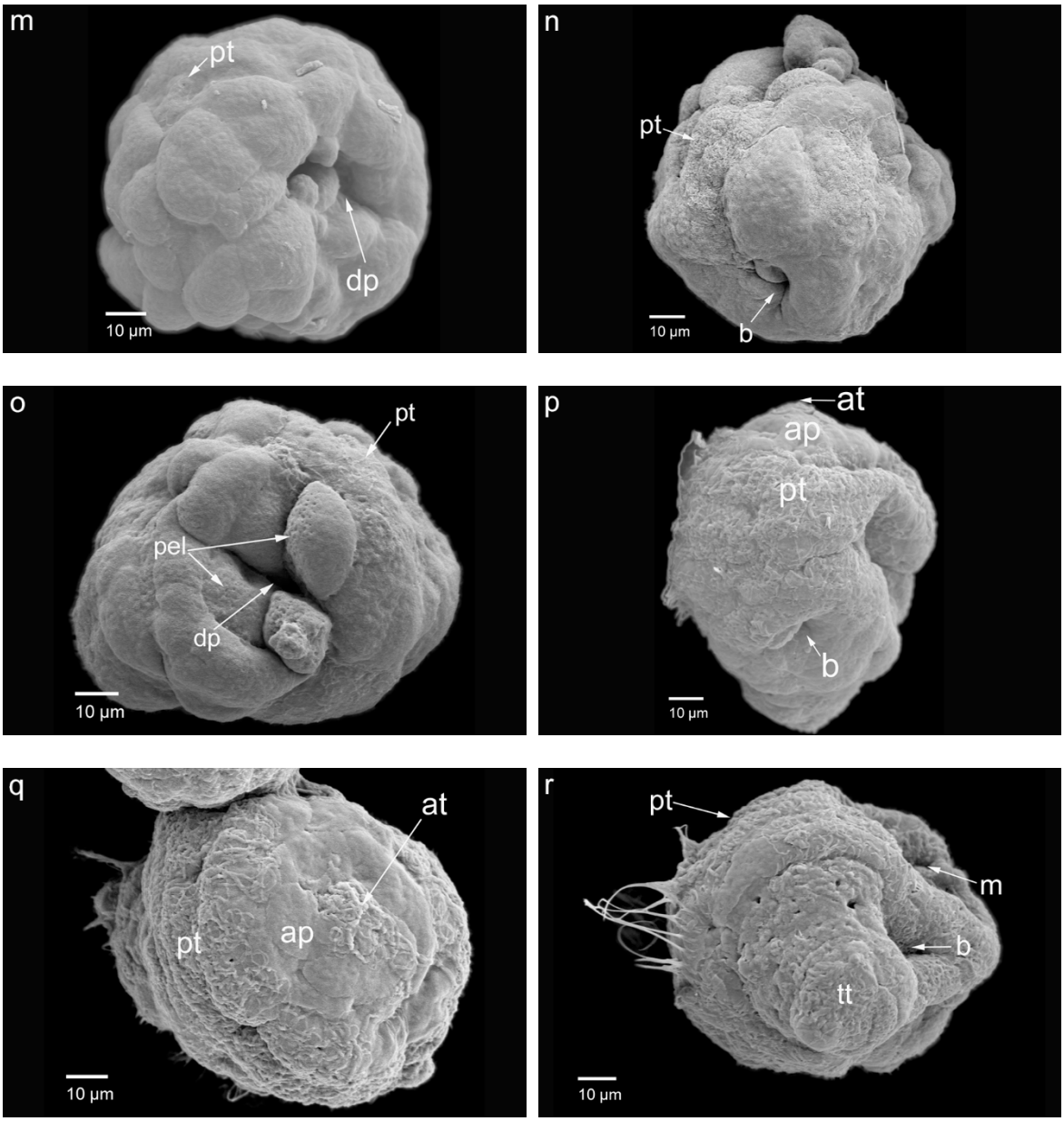


Figure 4.2 cont.

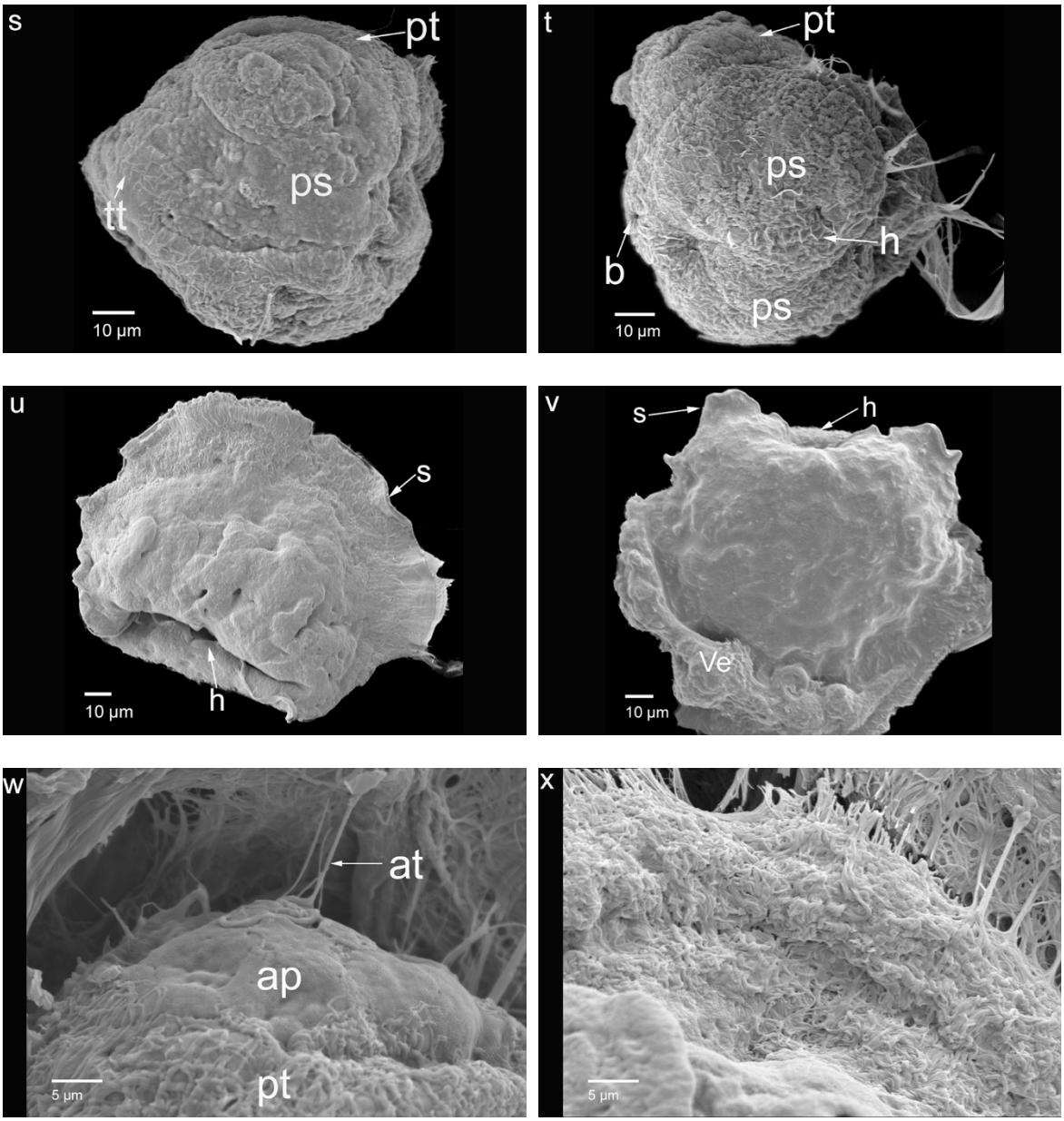
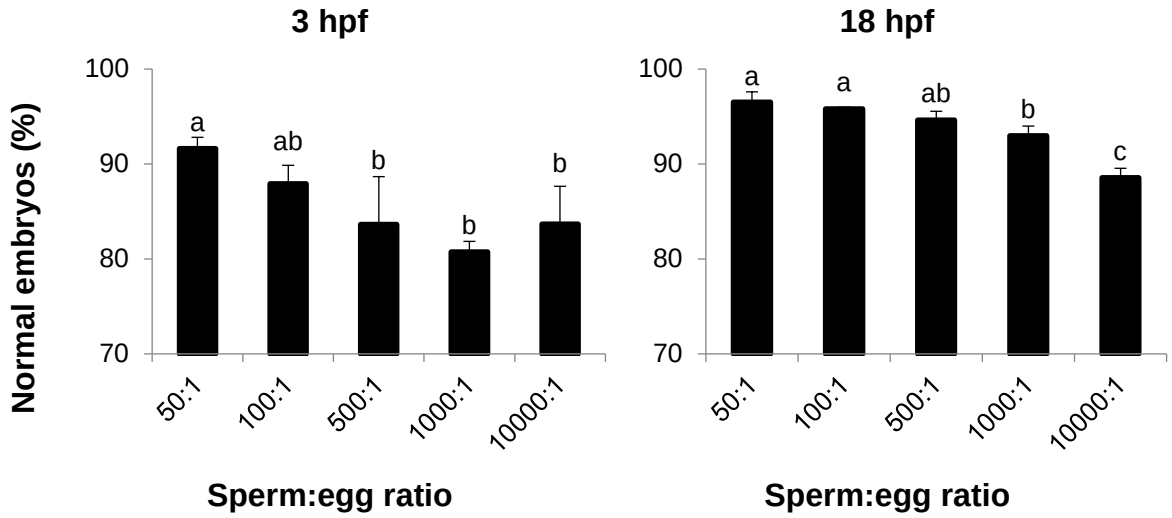


Figure 4.3 Proportion of apparently normal embryos, expressed as a mean percentage of initial egg numbers, 3 and 18 hpf using different sperm:egg ratio treatments. Bars represent mean percentage ± SD, n = 3; significant differences are identified by distinct letters ($P < 0.05$).



CHAPTER 5 – Functional morphology and performance of the New Zealand geoduck clam (*Panopea zelandica*) larvae reared in a flow-through culture system

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Abstract

Understanding the parameters required for a reliable hatchery seed production of the endemic geoduck clam *Panopea zelandica* (Quoy and Gaimard, 1835) is crucial if New Zealand is to develop a geoduck aquaculture industry. To provide the foundation for routine geoduck larval rearing practices, this study reports on physiological, morphological, and behavioral characteristics throughout the larval developmental process. *P. zelandica* larvae were reared at 17 °C and 35 ppt, and fed continuously with *Tisochrysis lutea* and *Chaetoceros calcitrans* in a flow-through system. The initial veliger stocking densities ranged from 50-200 larvae mL^{-1}, while residual algal levels were maintained at 20,000 to 80,000 cells mL^{-1} in three rearing batches. Larval behavior and morphology were described through observation using video recordings, photomicrographs and scanning electron microscopy. The larval development took 16-19 days from first D-veliger and metamorphosis occurred across a wide size range (300-375 µm shell length). The increase in shell length was linear over time and correlated with the deposition of striae in the prodissoconch II. The ingestion rate followed a power function with time and was closely correlated with the development of the alimentary system, including mouth, esophagus, stomach, style sac, intestine, and digestive gland. Rearing with an initial stocking density of 100 larvae mL^{-1} and residual algal background of 20,000 cells mL^{-1} resulted in about 76% survival and 15 µm d^{-1} growth rate. The results of the preliminary production trials inform rearing practices and provide biological descriptions that appear to be effective as a baseline protocol for the successful commercial production of *P. zelandica* larvae.

5.1 Introduction

Geoducks (*Panopea* spp.) are large clams with a global, but patchy distribution that is limited to a few countries, including USA, Canada, Mexico, Argentina, Italy, Spain, Japan, Korea, and New Zealand (Leyva-Valencia et al., 2015). They are among the largest living burrowing clams, with a long life span of up to 160 years and exceed 3 kg live weight (Goodwin and Pease, 1989). Although the anatomy of geoducks is broadly similar to that of other clam species, they have extremely long siphons, which allow them to irrigate their branchial chambers while buried down to 1 m below the sediment surface (Campbell et al., 1998). When retracted, the siphons do not fit within the two valves, preventing complete occlusion; the animal therefore relies on its deep infaunal lifestyle to escape predation (Goodwin and Pease, 1989).

Geoducks represent one of the most lucrative products in the seafood market, especially in Asia. For example, a top-graded live Pacific geoduck can be sold for USD$200 – 300 per kg in Asian restaurants (Shamshak and King, 2015). To meet this demand, the world geoduck production has increased from 3000 tons in 1997 to 6000 tons in 2008 (Shamshak and King, 2015). Historically, most of this production was based on wild harvests, but as wild stocks have been exploited to their maximal allowable catch (Zhang and Hand, 2006), aquaculture ventures have emerged throughout the world, especially in the USA and Canada (Feldman et al., 2004). In New Zealand, the endemic geoduck, *P. zelandica*, has been identified as a new species that can support the country's growth aspirations for the aquaculture sector to generate annual exports exceeding $1 billion in revenues by 2025 (Carter, 2002). However, in order to develop a sustainable New Zealand geoduck

aquaculture industry, a successful hatchery production of seed will need to be established (Gribben and Heasman, 2015).

One of the most important aspects of hatchery production is a good understanding of the larval biology of the target species (Shumway and Parsons, 2006). Without thorough groundings in the fundamental aspects of larval biology such as development, functional morphology, ingestion rates, growth and survival hatchery managers are not able to develop a good larval rearing procedure or understand why it is done in that way (Helm et al. 2004). Indeed, many practical hatchery manuals have been written for bivalves such as hard clam, oyster, and scallop based on the published information of their larval biology (Kraeuter and Castagna, 2001; Helm et al., 2004; Shumway and Parsons, 2006). Although previous studies have described aspects of larval development for *P. generosa* (Goodwin et al., 1979), *P. japonica* (Lee and Rho, 1997), *P. globosa* (Ferreira-Arrieta et al. 2015), and *P. zelandica* (Gribben and Hay, 2003), relatively little information is available for geoduck larval biology. Similar to other bivalve larvae, geoducks have different planktonic stages, which include a lecithotrophic trochophore, D-stage veliger, umbonate veliger and pediveliger (Goodwin et al., 1979; Lee and Rho, 1997; Ferreira-Arrieta et al., 2015; Gribben and Hay, 2003). Gribben and Hay (2003) demonstrated that *P. zelandica* D-larvae (105 µm mean shell length; SL) develop to 175 µm umbonate veligers in 9 days, then to 247 µm pediveligers within 15 days, and most larvae complete metamorphosis at 269 µm SL within 17 days at 17 ºC. The hinge structure of *P. zelandica* was also examined throughout the larval development (Gribben and Hay, 2003). However, these descriptions were limited to general morphology without functional interpretation

over different developmental stages. This fundamental knowledge is critical to the development of larval rearing techniques, such as management of stocking density, health assessment, feeding requirements and timing of metamorphosis.

To date, there are no detailed reports on the growth and survival of *P. zelandica* in hatchery conditions. However, studies have examined the growth and survival of other species of *Panopea* such as *P. generosa* and *P. globosa* under different densities and food rations in static systems (Marshall et al., 2014; Ferreira-Arrieta et al., 2015). The survival from D-veliger to pediveliger of *P. generosa* was shown in two studies to decrease with increasing stocking density (from 2 to 10 larvae mL^{-1}; Goodwin et al., 1979; Marshall et al., 2014). For example, the percent survival of *P. generosa* larvae decreased from 56% at 2 larvae mL^{-1} to just 7% at a density of 10 larvae mL^{-1} (Marshall et al., 2014). Ingestion rates have also been investigated in *P. generosa* and increased with increasing food ration from 5,000 to 100,000 cells $larvae^{-1}$ d^{-1} (Marshall et al., 2014). Similarly, the ingestion rate of *P. globosa*, which were stocked at 10-90 larvae mL^{-1} depending on size, increased with an increase in algal concentration from 50,000 to 100,000 cells mL^{-1} d^{-1}, but reached a plateau between 100,000 to 300,000 cells mL^{-1} d^{-1} (Ferreira-Arrieta et al., 2015). These previous studies on *P. generosa* (Marshall et al., 2014) and *P. globosa* (Ferreira-Arrieta et al., 2015) provided some guideline information for geoduck larval rearing in static systems. However, no studies have yet examined geoduck larvae performance in flow-through systems, which have been tested for other bivalve species (Magnesen et al., 2006; Rico-Villa et al., 2008; Ragg et al., 2010). The advantages of flow-through systems for rearing bivalve larvae are that the demand of labour and

space can be reduced (King et al., 2005); larvae may be reared at a high density (e.g. 200 larvae mL^{-1}, Ragg et al., 2010); food is provided at a constant density (Rico-Villa et al., 2009); and the constant water exchange continuously flushes waste products.

Thus, the aims of the present research are to characterize the relationship between the morphological form and its function in *P. zelandica* larvae throughout their developmental process, and to identify the growth, survival, and ingestion rates of geoduck larvae within a flow-through system. It is envisaged that this information will form the foundation for the development of hatchery-reared geoducks to seed an emerging New Zealand geoduck aquaculture industry.

5.2 Methods

5.2.1 Source of larvae

P. zelandica broodstock were collected from Golden Bay (South Island, New Zealand) and Bay of Plenty (North Island, New Zealand) between 2010 and 2014. These adults were conditioned in a semi-recirculating system and fed with phytoplankton from artificially fertilized eutrophic land-based seawater ponds for 9-10 months, then held at 15 °C and fed with *Tisochrysis lutea* and *Chaetoceros muelleri* for final conditioning for 2-3 months (Le et al., 2014). To induce spawning, a range of non-invasive methods previously used on this species were attempted, including thermal shock, addition of algae and addition of gametes (Gribben and Hay, 2003). None of these were successful for inducing *P. zelandica* to spawn, and thus serotonin injection was applied. About 30 animals were induced to spawn by injecting 1–2 mL of 2 mM serotonin into the mantle following a modification of the spawning induction method described in Gribben et al. (2014). Initial gamete

release allowed individuals to be sexed and then placed in separate seawater tanks. Gametes were collected within 30 min post-release, then pooled and stored at 4 °C for 0-2 h for sperm and 0-1 h for eggs. Fertilization was achieved at a sperm:egg ratio of 500:1 with approximately 200 eggs mL^{-1} based on an on-site optimized protocol established for the mussel *Perna canaliculus* (Adams et al., 2009). Embryos were then incubated in lightly aerated 170 L conical tanks containing 1 µm filtered seawater and 4 µmol EDTA at 17 °C for approximately 48 h, until 90% of surviving larvae had entered the D-veliger stage.

5.2.2 Larval rearing system

Newly-formed D-larvae were collected on a 45-µm sieve and transferred to a larval rearing system (Fig. 5.1), previously described by King et al. (2005) and Ragg et al. (2010). The system consisted of an array of bullet-shaped 2.5 L acrylic tanks, which received seawater and air through glass droppers (5 mm diameter). The seawater was delivered to the larval tanks by gravity from a header tank, which was filled with 1-µm filtered seawater and algae. An obliquely arranged polyethylene mesh screen (43 µm, 2 – 6 days post-fertilization; 55 µm, 7 – 9 dpf and 75 µm, > 9 dpf) with a high surface area was used to prevent larval washout.

5.2.3 Algal production

The microalgae *Tisochrysis lutea* (CS177, CSIRO, Tasmania, Australia; formerly known as *Isochrysis affinis galbana* or T-ISO clone; Bendif et al., 2013, 2014) were grown in continuous culture using 40 L bags (polyethylene; 15 cm diameter) supplied with 15 L d^{-1} of pasteurized seawater at 35 ppt salinity enriched with 0.1% (v/v) Conway medium. The microalgae *Chaetoceros calcitrans* (CS178, CSIRO, Tasmania, Australia) were grown in batch culture using 20 L polyethylene carboys,

which contained autoclaved 35 ppt seawater with a Conway diatom medium (0.1% v/v). All cultures received continuous aeration (15 L min^{-1}) supplemented with CO_2 (1% v/v) and were grown under a 24 h light regime (600 – 1000 lux) at 20 – 23 °C.

5.2.4 Rearing conditions

Three geoduck larval batches were produced in October 2010 (Batch 1), March 2014 (Batch 2), and October 2014 (Batch 3). The geoduck larvae were reared following an in-house operating procedure developed at the Cawthron Aquaculture Park (Cawthron Institute, Nelson). The purpose at this early stage of the emerging geoduck industry was to test how geoduck larvae performed with different protocols in the high density flow-through system. Consequently, different parameters were modified for each batch to initially test a variety of protocols. Briefly, larvae were reared in 1 µm filtered seawater at 17 ± 1 °C and 35 ppt. Water exchange of each larval tank was maintained at a rate of 80 mL min^{-1}. At 2 day intervals the seawater in each larval tank was drained through a sieve with a mesh size of 45-250 µm (depending on larval size), intended to remove detritus, empty shells, and small larvae (poor performers). Larvae which were retained on the mesh were rinsed into a 1000 mL beaker. Triplicate 100 µL samples were taken from this beaker to estimate the number of larvae after they were homogenously suspended by a nylon plunger. The remaining larvae were returned to their tanks. The geoduck larvae were fed with a mixture of *T. lutea* and *C. calcitrans* (1:2 ratio based on cell counts; following Ragg et al., 2010) which were continuously pumped from their culture vessels into the larval header tank by a pneumatic pump (SMC Pneumatics pump PB1011, Indianapolis, USA; Festo Pneumatics clear polyurethane tubing, 6 mm Φ, Esslingen, Germany). The

ingestion rates were determined 2 to 6 times daily, based on the algal concentration differences between the inflow and outflow of each tank (see below), to adjust the food input. Tanks and manifolds were rinsed with 4% hypochlorite bleach solution every two days while mesh guards were removed and rinsed with hot water daily. The larval rearing phase was deemed to be complete when ≥ 10% of individuals had metamorphosed into spat.

Algal concentrations were measured within the supply system to ensure that there was complete mixing. After corroborating that the algal concentrations were homogeneous, cell counts were made from outflow of each tank to establish residual background algal concentrations. Following the recommendations of Ragg et al. (2010) for mussel larvae, the residual algal background level was maintained at approximately 40,000 cells mL^{-1} for young larvae (2 – 10 dpf) and 80,000 cells mL^{-1} for older larvae (≥ 11 dpf) in Batch 1. The algal background level in Batch 2 was approximately 40,000 cells mL^{-1}, and in Batch 3 was approximately 20,000 cells mL^{-1} for the entire rearing period. In Batch 1, geoduck larvae were initially stocked at densities of 50 and 100 larvae mL^{-1} (n = 1). The initial larval density in Batch 2 was 200 larvae mL^{-1} (n = 2), while that in Batch 3 was 100 larvae mL^{-1} (n = 3).

One mL samples were taken daily from each tank to assess larval developmental stage, morphology, nutritional status and behavior. Larval activity and internal organ movements were video recorded for further behavioral analysis. Additional 1 mL larval samples from the tanks were fixed daily in Davidson's fixative at 4°C for further size measurement and morphological analysis under light microscopy and scanning electron microscopy. Approximately 30-50 larvae from

each tank were photographed daily (Olympus C7070 digital camera mounted on an Olympus CK2 inverted microscope at 40 times magnification). Images were analyzed using ImageJ software (version 1.40 g; http://rsb.info.nih.gov/). A customized particle recognition macro was used to isolate individual larvae and assess Feret's diameters (longest measurement across an ovoid), which was used as a measure of shell length. The larval growth rates were determined by the difference in shell length between days. The specific growth rates were calculated following the equation:

$$SGR = (ln(SL2) - ln(SL1))/t$$

where, SL1 and SL2 (μm) are the shell lengths at time T1 and T2 (day), respectively, and t is the time difference between T1 and T2 (days).

Larval survival was calculated as follows:

$$Survival = (Number\ of\ pediveligers\ /\ Initial\ number\ of\ D\text{-}larvae) \times 100\%$$

The in- and out-flow cell concentrations from each tank were measured with a Fluorimeter (Turner Designs, Sunnyvale, CA) or a Coulter Counter (Multisizer 4, Beckman Coulter). The Fluorimeter was used for rapid checks of feeding rates and when the Coulter Counter was not available. The Fluorimeter readings in mV were calibrated against the number of algal cells measured using the Coulter Counter. Ingestion rates were then estimated daily for each tank according to the equation:

$$I = V \times (\Delta C - \Delta C_{blank}) / P$$

where, I is the ingestion rate (cells larva^{-1} d^{-1}), V is the volume of flow-through seawater within a day (mL d^{-1}), ΔC is the difference in algal concentration between the inflow and outflow of the larval tank (cells mL^{-1}), ΔC_{blank} is the difference in algal concentration (cells mL^{-1}) between the inflow and outflow of a blank tank (containing no larvae), and P is the number of larvae, which is assumed to change linearly between assessment days.

5.2.5 Scanning electron microscopy

Larvae were removed from the Davidson's fixative and retained on a piece of 0.25 µm filter paper (Whatman® GF/C). They were then washed with a phosphate buffer (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4; pH = 7.4) for 5 min, followed by distilled deionized water for 1 min. Larvae then underwent a dehydration process of ascending series of ethanol (analytical grade) 50, 60, 70, 80, 90 and 100% for 15 min each (Turner and Boyle, 1974). After dehydration, samples were soaked in chloroform solution (analytical grade, 98%) before being dried in a desiccator. A filter paper soaked in chloroform was placed in the desiccator so that samples could dry within a vaporous environment overnight (Wassnig and Southgate, 2012). Once dry, samples were placed on adhesive carbon paper, which was attached to an aluminum stub. Samples were immediately sputter coated with carbon for 40 seconds using an ion sputter coater (Hitachi E 1045) before observation with SEM (Hitachi SU 70 Skottky). The background of the images was changed to black and their contrast and brightness were adjusted with Adobe Photoshop® CS6 software.

5.3 Results

5.3.1 Larval development

The shell-bearing stages of larval development in geoducks may be categorized into six stages: prodissoconch I D-veliger, prodissoconch II D-veliger, prodissoconch II early umbo veliger, prodissoconch II umbo veliger, pediveliger and metamorphosis. These stages are described in detail in the following section.

First stage: prodissoconch I D-veliger

The first shell-bearing stage is the prodissoconch I D-veliger, which has a straight hinge (Fig. 5.2a). These larvae are 2 days post-fertilization (dpf) with a shell length range of 100-105 µm. The prodissoconch I, which is secreted by the shell gland, has punctuated markings (Fig. 5.3a). The hinge is smooth without denticles (Fig. 5.3j). The internal organs can be observed through the transparent shell, as well as prominent extra-cellular lipid droplets distributed throughout the mantle region (Fig. 5.2b). The stomach, esophagus and retracted velum with cilia predominate in the internal space (Fig. 5.2a). When expanded, the velum presents a peripheral disk of cilia. There is an apical plate lying at the center of the disk. A single, long flagellum protrudes from the center of the apical plate and whips along the anterior posterior axis, apparently acting as a sensory organ (Carriker, 2001). This is a swimming larva, which retracts its velum when its flagellum comes in contact with another object, while re-expansion of the velum is preceded by the reappearance of the flagellum from between the partly opened shell valves. Larvae swim actively and do not close their valves after being exposed to ethanol, which indicates that nervous and/or sensory systems have not developed (e.g. *Crassostrea virginica*, Ellis and Kempf, 2011). Food collected by cilia within the

velum passes through the ciliated mouth and esophagus to a stomach. The stomach leads to a short, straight intestine opening to an anus, which is ciliated (Fig. 5.2a; 5.3n).

Second stage: prodissoconch II D-veliger

The second stage is the prodissoconch II D-veliger, which lasts from 3 to 6 or 7 dpf. Their shell lengths increase from 110-120 µm to 150-180 µm. On the third day, a narrow rim of the transition band from prodissoconch I to prodissoconch II shell (~10 µm) appears (Fig. 5.3a). Subsequently, the prodissoconch II, which is thicker than the prodissoconch I shell and bears concentric markings, is secreted by the outer fold of the mantle edge. The prodissoconch II is sculptured into minute commarginal growth striae. The first stria of the prodissoconch II is the largest (~20 µm), while the following striae are 7.5-9.0 µm (Fig. 5.3b). The hinge still lacks denticles (Fig. 5.3j), and the color of the larvae becomes light yellow. The mouth becomes more conspicuous and appears strap-shaped with well-developed cilia (Fig. 5.2c). The mouth leads to an esophagus, which is a densely ciliated tube. The esophagus runs dorsally into the visceral mass and opens into the anterior ventral region of the stomach. The rotation of food can be observed in the stomach. The stomach leads posteriorly to an intestine. The intestine begins to extend as a loop around the stomach and leads to the anus. The entire length of the stomach, intestine, and anus is ciliated (Fig. 5.2c). The mantle is conspicuous underneath the shell, and when the mantle is retracted into the shell, it occupies the ventral portion of the larva (Fig. 5.2c). The initial anterior adductor muscle is visible (Fig. 5.2c). Small lipid droplets can be seen around the visceral mass. The digestive gland changes color from light

yellow to orange-brown. Larvae swim actively and respond by closing their valves when being exposed to ethanol, which indicates that by this time sensory and/or neuro systems are developed (e.g. *Mytilus trossulus*, Voronezhskaya et al., 2008). Following a reactivity test (i.e. vibration of containers), the larvae stop swimming, and the 4 and 5 dpf larvae resume swimming after about 15-20 min, while older larvae swim again after 30 s to 2 min.

Third stage: prodissoconch II early umbo veliger

The third stage is the prodissoconch II early umbo veliger, from 6 or 7 dpf to 10 or 11 dpf. The larvae grow from 150-180 µm to 200-220 µm. The umbo rises slightly and uniformly above the hinge line. The new shell growth changes the shape of the D-larvae to round (Fig. 5.3b). The striae continue to be deposited on the prodissoconch II (Fig. 5.3b). An apical plate develops and is located dorsally to the velum (Fig. 5.2f; 5.3g). Apical ciliary tufts emerge from a shallow pit in the middle of the apical plate. The style sac protrudes dorsally from the fecal groove of the stomach. The style sac is a deep, cup-shaped organ consisting of densely ciliated cells (Fig. 5.2d). From the posterior end of the right side of the stomach, the intestine continues to extend and passes back around the end of the style sac, and then forwards to form an anterior loop lying to the left side of the stomach (Fig. 5.2d). The intestine runs dorsally beside the posterior adductor muscle to the anus (Fig. 5.2f). Hence, the stomach joins the esophagus at its ventral apex, the style sac at its flared dorsal base, and the digestive gland lobes posteriorly near their dorsal base. The digestive gland is an H-shaped organ, and its paired lobes connect through the central bar of the H with the stomach. The more prominent lobes of the digestive gland extend ventrally around the esophagus. The digestive

gland is found within both right and left valves, while the stomach appears only by the right valve. The feces are more conspicuous since more food is ingested. A fecal line was formed in the fecal groove at the base of the stomach and along the intestine (Fig. 5.2e). The intestine begins parallel with the frontal plane and on the right-hand aspect of the mid-sagittal plane. It then turns dorsally and curves 180° around the style sac, proceeding on the left-hand side of the mid-sagittal plane toward the anterior, ventral region of the visceral cavity. The intestine proceeds to a point approximately between the ventral tips of the digestive gland lobes and the anterior adductor muscle. There, it makes another 180° turn and proceeds in a posterior dorsal direction to the anus, which is located between the posterior adductor muscle and the mantle lobes. The lipid droplets become bigger and distribute around the digestive gland, intestine, and stomach (Fig. 5.2e). Dense glycogen granules (Fig. 5.2 g) surround the lipid droplets (e.g. *C. virginica*, Elston, 1980).

Fourth stage: prodissoconch II umbo veliger

The fourth stage is the prodissoconch II umbo veliger from 10 or 11 dpf to 15 or 16 dpf. The shell lengths increase from 200-220 µm to 320-350 µm. Small denticles are formed (Fig. 5.3k). A prominent foot develops. At first, the base of the foot develops as a thickening at the posterior side of the esophagus to become a trunk (Fig. 5.2e). Then, a byssal spur, statocysts and propodium appear (Fig. 5.2f). The pedal structure of the foot continues to develop with the extension of the trunk into the mantle cavity (Fig. 5.2f). The foot appears to be ciliated primarily along its posterior median axis. At the same time, the primordial gills become visible (Fig. 5.2e). The ctenidial development begins with the appearance of paired

tissue outgrowths near the areas of lateral fusion between the visceral mass and mantle on the posterior side. Then, four transverse columns of papillae develop on both sides of the foot (Fig. 5.2h and m). The anterior-most pair of papillae is bigger than the other posterior papillae (Fig. 5.2h and i). The anterior-most papillae are fused with the visceral epidermis along their anterior surface, while the other papillae extend freely into the mantle chamber (Fig. 5.2h and i). The posterior adductor muscle is apparent (Fig. 5.2f).

Fifth stage: pediveliger

The fifth stage is the pediveliger from 15 or 16 dpf to 19 or 20 dpf. The shell length ranges from 300 to 375 μm. The flanges appear at two sides of the dorsal ends of the shells (Fig. 5.3d and m). Both small and large denticles develop on the hinge (Fig. 5.3n). Ctenidial papillae elongate concurrently with the growth of cilia on their surfaces (Fig. 5.2h). The papillae continue to elongate and become primary filaments with dense lateral cilia. Before metamorphosis, a new papilla forms posteriorly by transverse divisions of paired rudiments. Hence, there are five primary filaments, and one papilla on the inner demibranchs. The cilia on the filaments create periodic water currents. The foot elongates to a point where the propodium can no longer be accommodated next to the mouth and straightens towards the ventral area when the valves are closed. The propodium protrudes from the viscera-pedal mass towards the edge of the posterior mantle cavity and its head folds back to the visceral cavity (Fig. 5.2h). Larvae still swim with their velum (Fig. 5.3h). When larvae do stop swimming, both their velum and foot protrude through the valves. The foot partially extends so that the propodium can make contact with the seawater or substrate.

Sixth stage: metamorphosis

The sixth stage is metamorphosis which is typically completed within 2 days (17-18 dpf or 21-22 dpf). Before metamorphosis, geoduck larvae swim up to the surface of the static water and aggregate together with mucous secretions. Swimming decreases markedly and eventually ceases, and larvae begin to settle. The velum is fully everted. The cilia are rounded and progressively shed from the velum, but some may be captured and ingested (Figs. 5.2k and l; 5.3i and f). The velar and anterior pedal retractor muscles contract rapidly and repetitively. These contractions pull dorsally and rotate anteriorly the apical plate, mouth, esophagus, viscera and foot. This rotation can be marked by the orientation of the apical turf (Fig. 5.2i). The apical plate migrates near the anterior adductor muscle. The foot migrates anteriorly, displacing the posterior velum and esophagus. The siphon septum forms by local, posterior expansion of the inner mantle folds near the ctenidial rudiment (Fig. 5.2j). Each mantle lobe develops cone-like projections, which fuse to form a thin tissue septum. The propodium abrades the velum and esophagus, potentially aiding dissociation of the velar margin. The fused apical plate and esophagus detach from the marginal sub-velar tissues. The larval mouth is lost, but still attached to the marginal sub-velar tissues. The remaining sub-velar marginal tissues are absorbed by the mantle. At this stage, the animals are termed spat (Fig. 5.2m) and they develop dissoconch shells, which are covered with spines (Fig. 5.3e). These spines (max. ~ 5 µm length) are also seen both inside and outside the shell edge (Fig. 5.3f).

5.3.2 Larval performance

Survival during the first five days of the veliger cycle (2-6 dpf) ranged from 60.1% (Batch 2) to 95% for Batch 1 (Fig. 5.4a). The relative survival to 20 dpf (based on the number of surviving larvae at 6 dpf) ranged from 54.8% (Batch 2) to 81.9 ± 6.2% for Batch 3 (Fig. 5.4b).

The growth in shell length of geoduck larvae in each batch was linear over time and can be described as:

$SL = 16.23 \times t + 75.49$ ($R^2 = 0.98$) in Batch 1 at 100 larvae mL^{-1},

$SL = 15.80 \times t + 76.89$ ($R^2 = 0.99$) in Batch 1 at 50 larvae mL^{-1},

$SL = 10.36 \times t + 93.6$ ($R^2 = 0.99$) in Batch 2 at 200 larvae mL^{-1}, and

$SL = 15.28 \times t + 76.47$ ($R^2 = 0.98$) in Batch 3 at 100 larvae mL^{-1}

where, SL is the shell length (µm) and t is the number of days of culture (day 1 = 2 dpf; Fig. 5.5).

Specific growth rates typically ranged from 10.7 to 14.4% d^{-1} during the first 2-3 dpf, decreasing to 7.4 to 9% d^{-1} for the remainder of the larval rearing period. The mean gross growth rate during the larval rearing period (µm d^{-1} shell length gain) ranged from 10.36 to 16.23 µm d^{-1}.

The ingestion rate of geoduck larvae followed a power function with time as:

$IR = 552.76 \times e^{0.2315 \times t}$ ($R^2 = 0.88$) in Batch 1 at 100 larvae mL^{-1},

$IR = 453.21 \times e^{0.2450 \times t}$ ($R^2 = 0.77$) in Batch 1 at 50 larvae mL^{-1},

$IR = 1968 \times e^{0.1214 \times t}$ ($R^2 = 0.82$) in Batch 2 at 200 larvae mL^{-1}, and

$IR = 922.51 \times e^{0.2296 \times t}$ ($R^2 = 0.93$) in Batch 3 at 100 larvae mL^{-1}

where, IR is the ingestion rate (cells $larva^{-1}$ d^{-1}) and t is the number of days of culture (day 1 = 2 dpf; Fig. 5.8).

Ingestion rates 4 dpf ranged from 295 (Batch 1) to 1763 (Batch 3) cells $larva^{-1}$ d^{-1}, increasing exponentially during the following 12 – 16 days, reaching 12056 (Batch 2) to 35994 (Batch 1 at 100 larvae mL^{-1}) cells $larva^{-1}$ d^{-1} (Fig. 5.8).

5.4 Discussion

5.4.1 Larval development

The larval development of *P. zelandica* in our study (at 17 ºC) took 16-19 days from first veliger, and metamorphosis occurred at about 18 – 22 dpf across a wide size range (300-375 µm). Despite the same rearing temperature, the *P. zelandica* larvae in the present study exhibited a longer larval period and metamorphosed at larger sizes than those described by Gribben and Hay (2003), which metamorphosed at 15 days and 247 µm. In addition, Gribben and Hay (2003) did not observe any spat after 2 days of setting, indicating incomplete metamorphosis. The *P. zelandica* larval rearing period, in the present study, was longer than that of the geoduck *P. globosa* reared at 22 °C (13-14 days), but with a similar size at metamorphosis (343 µm; Ferreira-Arrieta et al., 2015). In contrast, the larval period in the current study was shorter than that of the geoduck *P. generosa* reared at 17 °C (16-35 days), and the larvae were smaller in size at metamorphosis (350-400 µm; Goodwin and Pease, 1989).

The present study provides a detailed description of *P. zelandica* organogenesis, including shell, mantle and mantle cavity, alimentary canal, ctenidia, musculature, foot and byssal glands. The observation of larval shell morphology (prodissoconch I and II) of *P. zelandica* in our study are in agreement with, and add detail to the description in Gribben and Hay (2003). Additionally, we include descriptions of the shell morphology of spat (dissoconch and spines), that augment those in previous studies. The shell morphology of *P. zelandica* was similar to that of *P. generosa* (Goodwin et al., 1979). The SEM images, in the present study, illustrate how striae are deposited across different stages. The larval organogenesis of *P. zelandica* was similar to that of *P. generosa*, which was observed by histological sections (Bower and Blackbourn, 2003). We also report on the specific developmental processes that operate and interact to achieve the functional and specialized requirements of larvae at each developmental stage. These findings suggest that larval organogenesis of *P. zelandica* is similar to that of other geoducks (Bower and Blackbourn, 2003) and related clams, such as *Venerupis striatula* and *Mercenaria mercenaria* (Ansell, 1962; Carriker, 2001).

5.4.2 Functional morphology

The larval growth rates in this study were consistent with the observed morphological development of larval shells throughout the larval period. When the first and largest stria of the prodissoconch II was deposited, the highest specific growth rates were encountered (e.g. Figs 5.3b and 5.7). The subsequent striae, which were smaller than the first, but had a similar size to each other, were secreted daily; hence, from this point the larval size increased as a linear function of time. Unlike growth, the ingestion rate of *P. zelandica* larvae did not follow a

linear function, but a power function, which was in agreement with the development of the alimentary system. The incomplete alimentary canal with small velum band, narrow esophagus, small stomach, and short intestine resulted in a low ingestion rate during 2-5 dpf (first and second stage). The ingestion rate increased dramatically at the umbo veliger and pediveliger stages (fourth and fifth stage). This marked increase may have been due to the full development of the alimentary canal including velar cilia, esophagus, stomach, crystalline style, gastric shield, style sac, digestive gland, intestine and anus (Bower and Blackbourn, 2003), which was observed at these stages. Thus, it is conceivable that the shell and alimentary system play an important function for the larval survival and growth.

Integument

The normal growth of larval shells and their integrity are vital for larval survival (Talmage and Gobler, 2010). Bivalve shells have several main functions such as protection from predators and suspended particles (Carriker, 1996), support for muscular attachment (Kasyanov et al., 1998) and other soft and delicate internal organs (Carriker, 1986). Hence, shell malformation or deformation may interfere with the regular development of a larva. For example, if the prodissoconch I is too small compared to the whole larva, the velum will not be encompassed within the two valves, consequently leaving the soft tissues unprotected (e.g. *Mytilus galloprovincialis*, Schönitzer and Weiss, 2007). In addition, a shell with a wavy edge (see Supplementary 5.1: S1, S2) prevents complete valve closure (Schönitzer and Weiss, 2007; Supplementary 5.1: S3). A damaged shell can also contribute to development of asymmetric valves (Supplementary 5.1: S4, S5) as the geoduck larva grows. Deformation and malformation of the shells and hard

parts (e.g. denticle) can affect the swimming and feeding ability of bivalve larvae, consequently reducing the growth and survival (Doney et al., 2009; Talmage and Gobler, 2010).

Feeding

The ciliation of the velum plays a vital role in the feeding function of bivalve larvae (Cragg, 2006). From the sagittal view the continuous beat of the preoral cirri of *P. zelandica* larvae creates metachronal waves to sweep particles towards the adoral cirri band (Supplementary 5.2: V1). The postoral cirri beat in the opposing direction to the preoral cirri deflecting the particles into the adoral tract. The adoral cirri then carry the particles towards the mouth where they are either ingested or rejected by the ciliated mouth and esophagus. The cilia band arrangement of *P. zelandica* larvae is comparable with that of other bivalves such as Pacific oyster *Crassostrea gigas* (Strathmann and Leise, 1979), scallop *Pecten maximus* (Cragg, 1989), and clam *M. mercenaria* (Gallager, 1988) which also have preoral, adoral and postoral bands of cilia. This arrangement allows planktonic bivalve larvae to feed on algae following the 'opposed band' capturing mechanism (Strathmann and Leise, 1979) or downstream collection (Riisgård et al., 2000). Consequently, the feeding capacity of planktonic geoduck larvae increases when the elongation and compounding of the cilia on the velum are advanced. The complete development of the digestive system also contributes to the increase in feeding capacity. For example, more food particles were observed being rotated in the stomach of *P. zelandica* larvae at later stages. The expansion of the stomach and the thickening of the inner lining of stomach allows more food to be rotated and digested (e.g. *Aequipecten irradians concentricus*, Sastry, 1965). In addition, the appearance of

a style sac and crystalline style, and the enlargement of digestive gland at later larval stages of *P. zelandica* might enhance enzyme production as in other bivalve species (e.g. *M. mercenaria*, Grizzle et al., 2001), consequently increasing nutrient assimilation and storage.

A similar feeding rate pattern during larval development was seen in another geoduck species, *P. globosa* (Ferreira-Arrieta et al., 2015) with the sharp increase in umbo veliger and pediveliger stages. Similarly, when the mussel *P. canaliculus* and the oyster *C. gigas* larvae change from mixotrophic to exotrophic stage, in which the parental energy is depleted and the energy is sourced completely from external food, their ingestion rates increased sharply (Ragg et al., 2010; Rico-Villa et al., 2009). The increase in feeding rate at the fourth and fifth stage of geoduck larval development is associated with the accumulation of nutrient reserves before metamorphosing into spat (Helm et al., 2004). These nutrient reserves are critical for newly metamorphosed spat survival since the velum (larval feeding apparatus) is lost, but the gills (newly feeding apparatus) are not yet fully developed (Gui et al., 2016) and are unable to capture algae (Supplementary 5.2: V2). At the same time, young spat are required to expend energy on activities such as shell growth and crawling. Hence, providing sufficient food for geoduck larvae at the fourth and fifth stage is paramount to fuel metamorphosis and early spat survival.

5.4.3 Practical husbandry

The larval performance results in this study display the variation that is to be expected among different larval batches (e.g. Helm et al., 2004) for a given species. While the batches reported here were reared under a range of conditions and unreplicated in one batch, they illustrate that geoduck larvae can also be

reared to settlement at much higher densities than those reported in previous studies (Table 5.2) using the continuous rearing system. Our results of high density larval culture were compatible with those reported for other shellfish species using similar systems (Ragg et al., 2010; Rico-Villa et al., 2009). Hence, the approaches trialed in the present study provide an effective baseline for further systematic assessment of the parameters influencing larval geoduck performance.

General guidelines can be made from our preliminary production trials regarding the physiological, morphological and behavioral characteristics that may be expected as a result of routine larval rearing practices. While survival 2-6 dpf ranged from 60-95%, all except Batch 2 showed 95% survival over this period. This suggests that the rearing conditions were adequate during this period, if not optimal. As these were early production trials, and not all parameters were held equal between Batches, it is not possible to draw conclusions regarding the effect of elevated density (200 larvae mL^{-1}) on the reduced survival in Batch 2. The initial stocking density in Batch 2 was deliberately elevated, at 200 larvae mL^{-1}, to correspond with recommendations made for *P. canaliculus* larvae under similar conditions (Ragg et al., 2010), in contrast to those for *P. maximus* of 3 – 12 larvae mL^{-1} (Magnesen et al., 2006). The densities in Magnesen et al. (2006) suggest that low survival before early umbo stage can take place at any density. Indeed, the survival of the Manila clam *Ruditapes philippinarum* larvae during the first 10 days was not affected by densities of 5 – 20 larvae mL^{-1} (Yan et al., 2006). The larval survival of the clam *M. meretrix* was also independent of stocking density (Liu et al., 2006). Hence, it is possible that a density of 200 larvae mL^{-1} may not be solely responsible for the low survival during the first five days in Batch 2.

Adequate larval performance was achieved over the range of food rations reported here. Similarly, feeding has not been critical for larval survival during the first few days of culture in previous bivalve studies (e.g. Carriker, 2001; Tang et al., 2006; Matias et al., 2011). For example, the survival of the larval clam *M. meretrix* was not affected by delayed feeding for the first 4 days of culture (Tang et al., 2006). Also, the survivorship of the starved larvae of the clam *R. decussatus* was high until day 8 of culture (Matias et al., 2011). Larvae of the oyster *C. gigas* and mussel *P. canaliculus*, which were reared with an algal background level of 20,000 and 40,000 cells mL^{-1} also had similar survival rates until the pediveliger stage (Rico-Villa et al., 2009; Ragg et al., 2010).

Excessive *C. calcitrans* may result in increased transparent carbohydrate exopolymers (Corzo et al., 2000), which can result in cell clumping and adhesion to surfaces (Ragg et al., 2010), thus promoting bacterial loading (Kormas, 2005). A monospecific diet of *C. calcitrans* produced adequate growth in *C. gigas* larvae, but led to high survival variability and low larval competence and metamorphosis (Rico-Villa et al., 2006). Similarly, a high algal concentration of 120,000 cells mL^{-1} of a bispecific diet of *T. lutea* and *C. calcitrans* resulted in low survival of *P. canaliculus* larvae (Ragg et al., 2010). Moreover, in a static system, geoduck *P. generosa* larvae had decreased survival with increasing food rations (Marshall et al., 2014). Hence, further research is needed to address the effect of food rations on survival of *P. zelandica* larvae, including the elucidation of the tradeoffs between food rations and deleterious side-effects associated with excessive residual algal cell numbers.

It was observed that the proportion of deformed larvae in Batch 2 was higher than that of the other batches. The deformed larvae had cracked shells, abnormal shape and small vela. While these deformed larvae survived, they did not grow, and were screened off, resulting in the low apparent survival rate in Batch 2 within the first five days (2-6 dpf). Therefore, the survival of geoduck larvae during the first five days of culture (2-6 dpf) may be affected by D-larval health rather than density or food rations. In addition, the fertilization kinetics of *P. zelandica* were seen to substantially influence the degree of polyspermy, potentially causing deformity of subsequent D-larvae (Gribben et al., 2014). Very high sperm:egg ratios observed during part of the Batch 2 spawning may have induced these conditions.

In conclusion, *P. zelandica* larvae can be effectively cultivated using flow-through systems with suggested stocking densities up to 100 larvae mL^{-1} and continuous algal feeding, maintaining a background level of 20,000 cells mL^{-1}. Along with larval performance, the morphological development and organogenesis of *P. zelandica* larvae are extremely important for hatchery operators to monitor daily husbandry. The present trials did not offer compelling evidence to suggest that stocking densities and algal background levels substantially influenced growth and survival of *P. zelandica* larvae within the parameters used in this study (50 – 200 larvae mL^{-1}; 20,000 – 80,000 algal cells mL^{-1}). Results of growth and survival also suggest that *P. zelandica* larvae can be potentially reared at high densities in a flow-through system (≥ 200 mL^{-1}), but further corroboration is required. The viability of high-performance, high density rearing systems may also be enhanced by carefully refined fertilization strategies, which are currently under investigation

as part of an ongoing program to develop a New Zealand geoduck aquaculture industry.

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Table 5.1 List of abbreviations used in Figure 5.2 and 5.3

a	anus	p	propodium
aam	anterior adductor muscle	pam	posterior adductor muscle
ac	anal cilia	pc	propodium cilia
ado	adoral cilia	pft	presumptive foot
ap	apical tuft	PI	prodissoconch I
apm	apical margin	PII	prodissoconch II
at	apical tuft	pre	preoral cilia
bg	byssal gland	ps	punctuate stellate
Ci	cilia	pso	postoral cilia
Cis	shed cilia	pt	propodium cilia tuft
Dg	digestive gland	r	rectum
dgg	dense glycogen granules	S	stomach
DIS	Dissoconch	sc	statocyst
e	esophagus	sd	small denticles
f	feces	sis	siphon septum
fc	filamentary cilia	sit	siphon tentacle
fg	fecal groove	Sp	spine
fla	flange	SS	style sac
gr	gill rudiment	um	umbo
H	Hinge	Ve	velum
i	Intestine	vm	viscera mass
il	intestinal loop	arrow head (black)	retractor muscle
ld	large denticles	arrow head (white)	stria
M	mouth	arrow head (yellow)	lipid droplet
mc	mantle cavity	#	boundary PI/PII
mf	mantle fold	##	bounday PII/dissoconch
mp	mantle papilla	* (black)	pair of papillae
npc	nonphagocytic free cell	* (white)	largest pair of papillae

Table 5.2 A summary of maximal growth rate of geoduck larvae reared in different conditions

Species	Temperature (°C)	Initial stocking density (larvae mL^{-1})	Algal species	Feeding ration (10^3 cells mL^{-1})	System	Maximal growth rate (μm d^{-1})	References
Panopea generosa	14 ± 1	4 - 10	*Diacronema lutheri**, *Tisochrysis lutea*, *Pseudoisochrysis paradoxa*, and *Phaeodactylum tricornutum*	50	650 L rectangular static tank	5.7	Goodwin et al., 1979
P. generosa	13.7 ± 0.24	2	*T. lutea*	10 - 200	1 L static jar	3.4	Marshall et al., 2014
		5	*T. lutea*	25 - 500		3.85	
		10	*T. lutea*	50 - 1,000		3.79	
P. globosa	22 ± 1	5	*T. lutea*	5 - 60	500 L conical static tank	21.9	Ferreira-Arrieta et al., 2015
P. zelandica	17 ± 1	6	*T. lutea*, *Thalassiosira pseudonana*, and *Diacronema lutheri*	15	2000 L static tank	10	Gribben and Hay, 2003
P. zelandica	17 ± 1	50 - 200	*T. lutea* and *Chaetoceros calcitrans*	20 - 80	2.5 L flow-through bullet tank	15	This study

* *Diacronema lutheri* was previously known as *Pavlova lutheri* or *Monochrysis lutheri* (Bendif et al., 2011)

Figure 5.1 Schematic of the flow-through larval rearing system (after King et al., 2005 and Ragg et al., 2010).

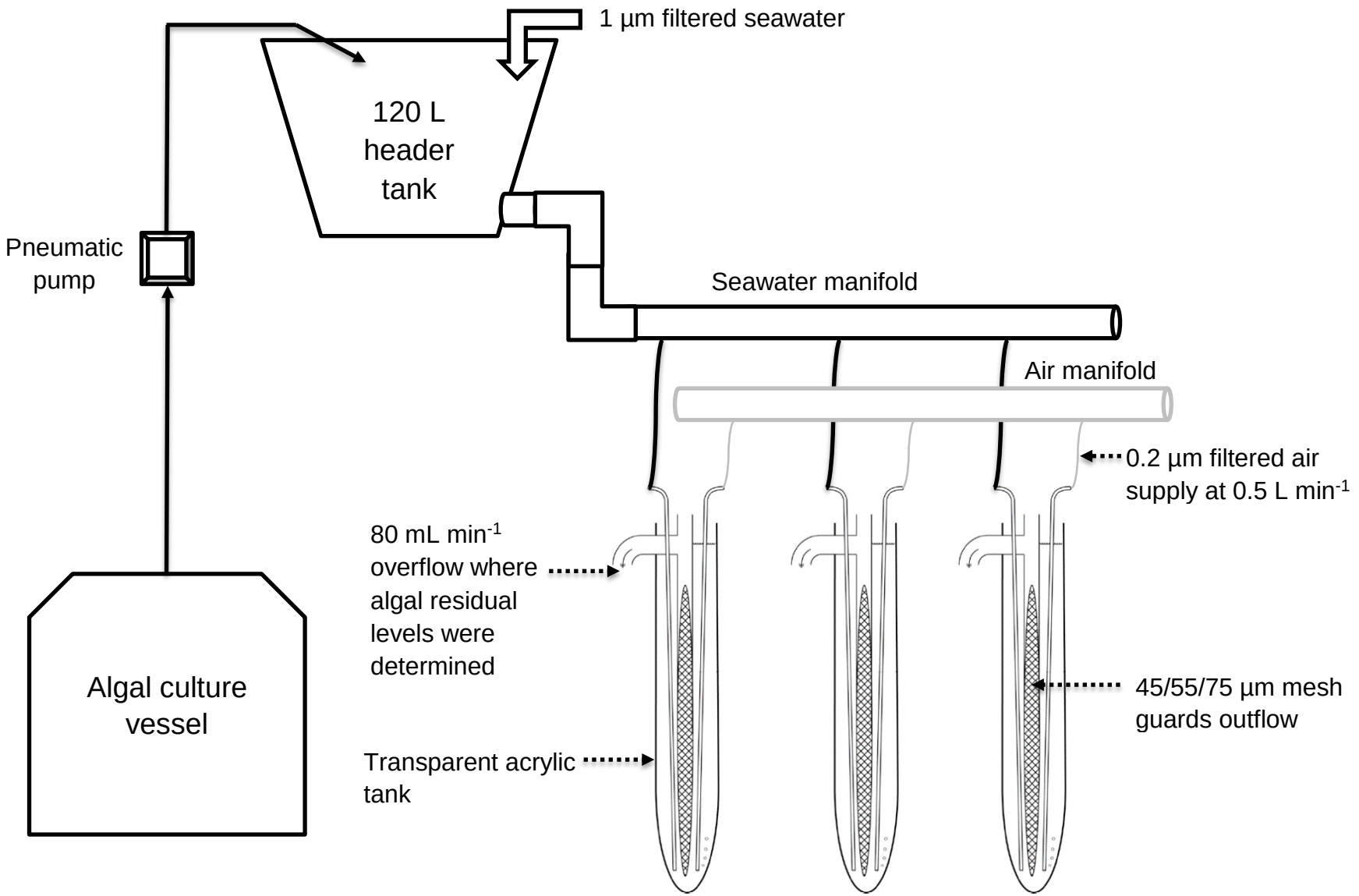

Figure 5.2 Larval development of *P. zelandica*. Light scope micrographs. a, b) Stage 1: prodissoconch I D-veliger. c) Stage 2: prodissoconch II D-veliger. d) Stage 3: prodissoconch II early umbo veliger. e) Stage 4: prodissoconch II umbo veliger. f) Stage 5: pediveliger. g to m) Stage 6: metamorphosis. Abbreviations are summarized in Table 5.1.

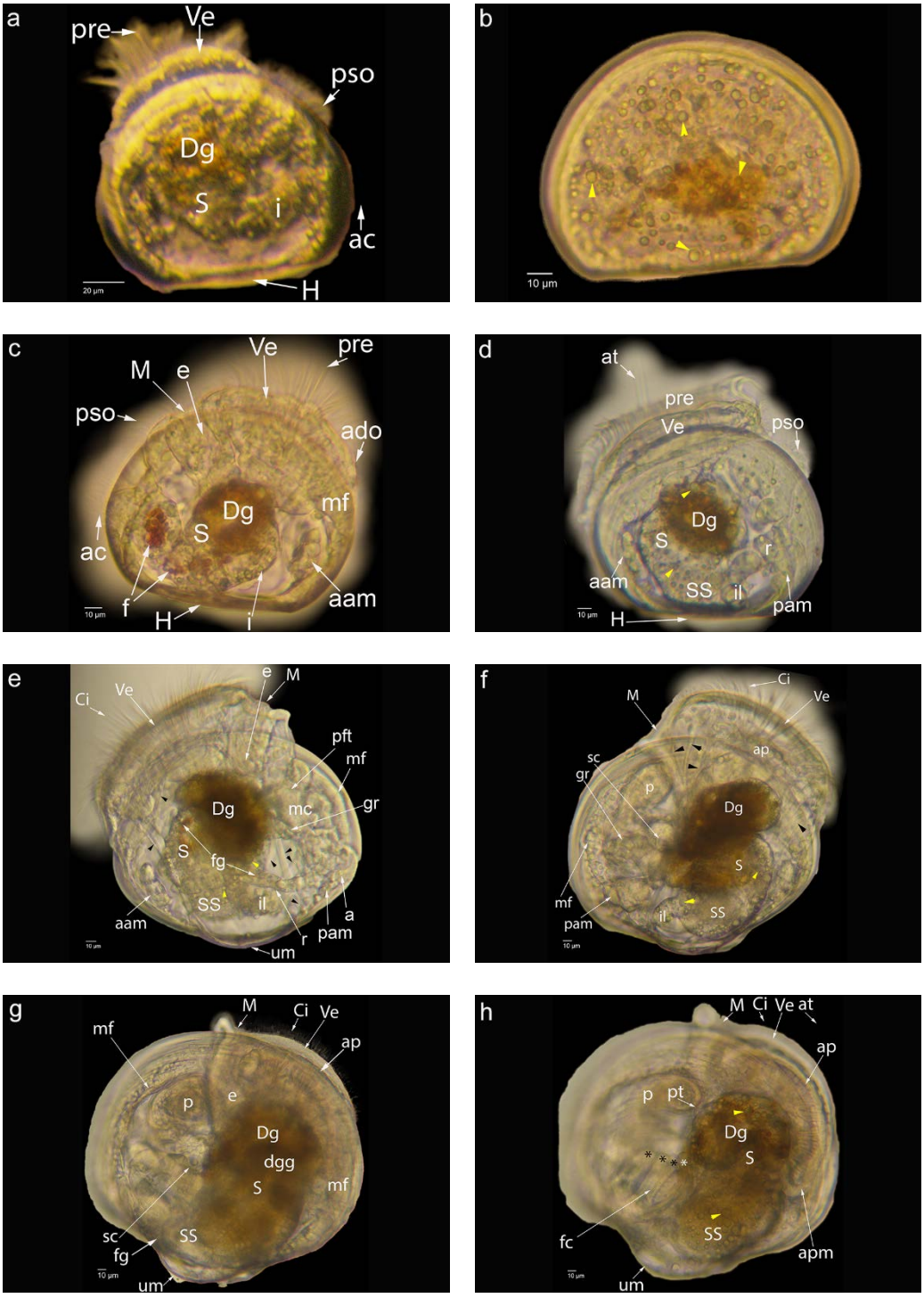
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Figure 5.2 cont.

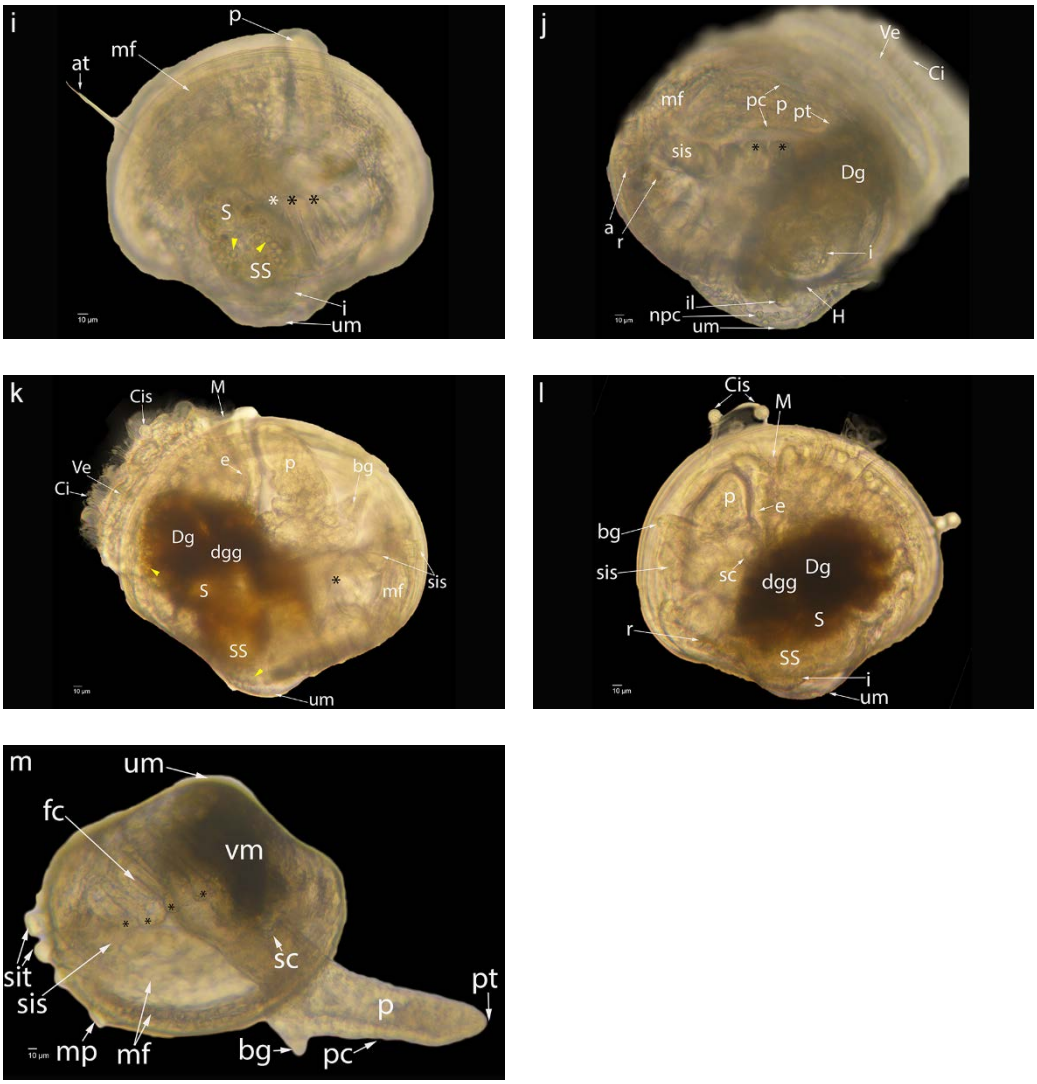

Figure 5.3 Larval development of *P. zelandica*. Scanning electron microscopy. a, j) Stage 2: prodissoconch II D-veliger. b, n) Stage 3: prodissoconch II early umbo veliger. c, g, k) Stage 4: prodissoconch II umbo veliger. d, h, l, m) Stage 5: pediveliger. e, f, i) Stage 6: metamorphosis. a to e) whole animal, f) shell margin, g to i) velum and cilia, k to l) internal hinge region, m) external hinge region, n) anal region. Abbreviations are summarized in Table 5.1.

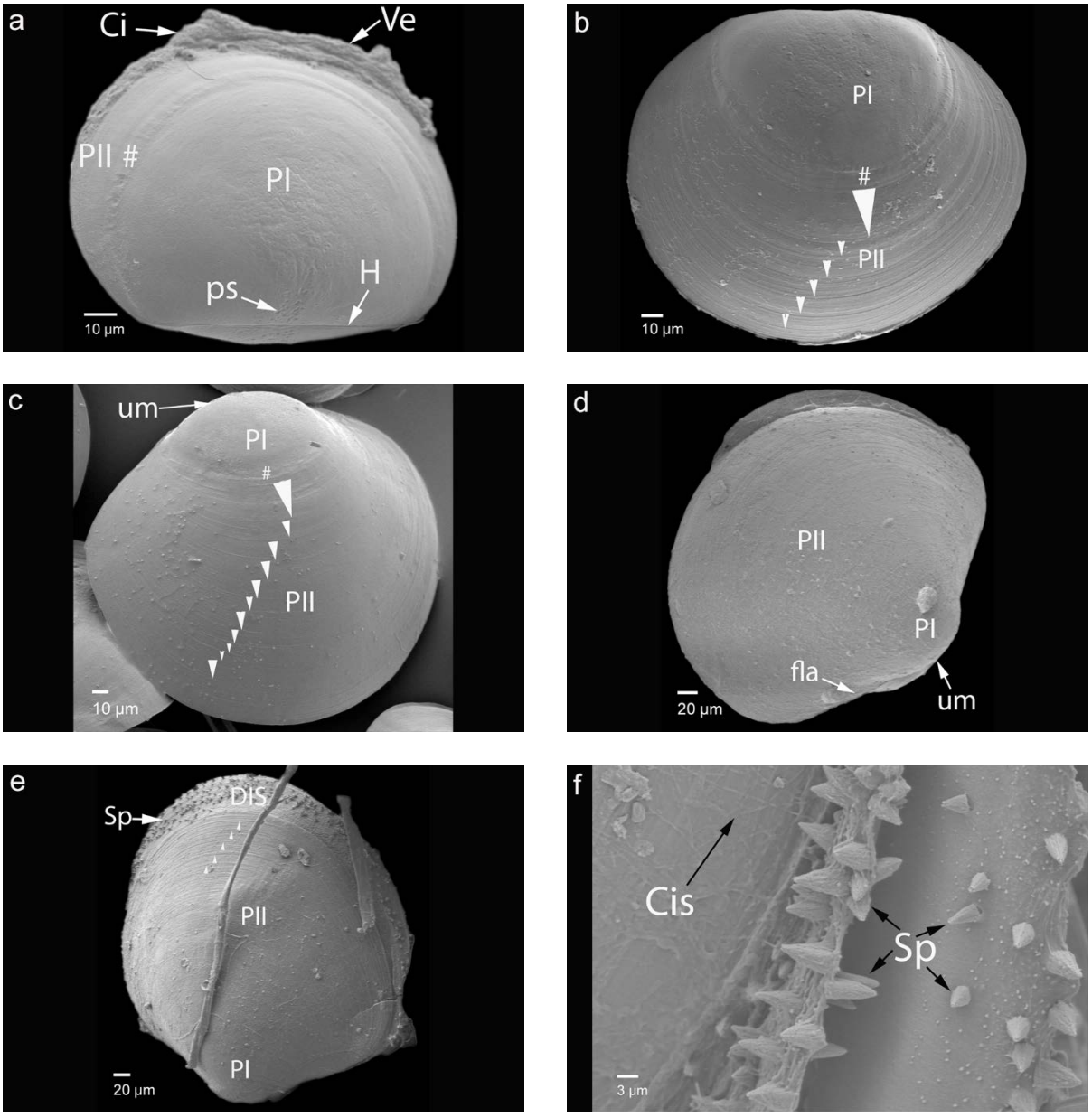
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Figure 5.3 cont.

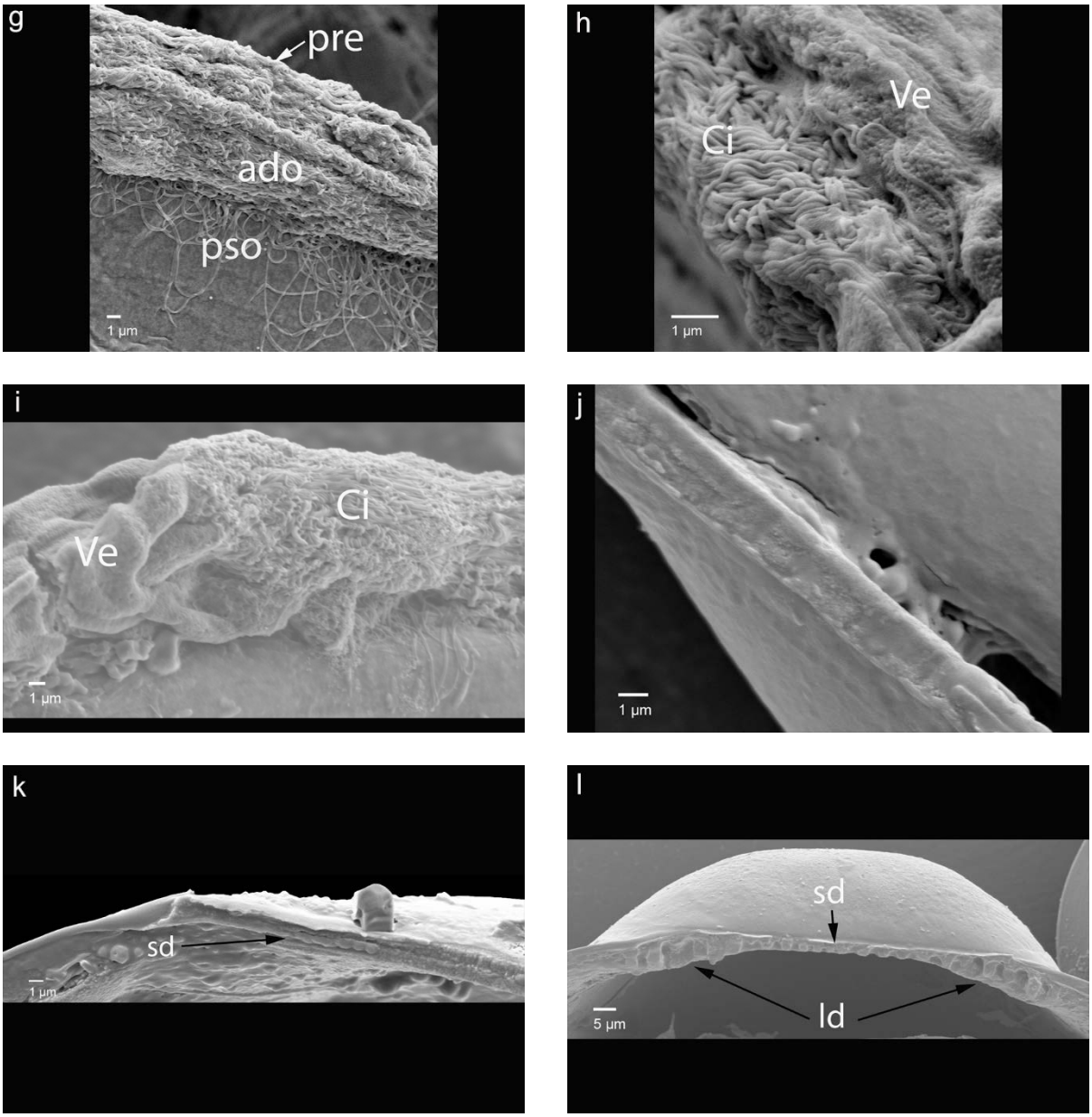


Figure 5.3 cont.

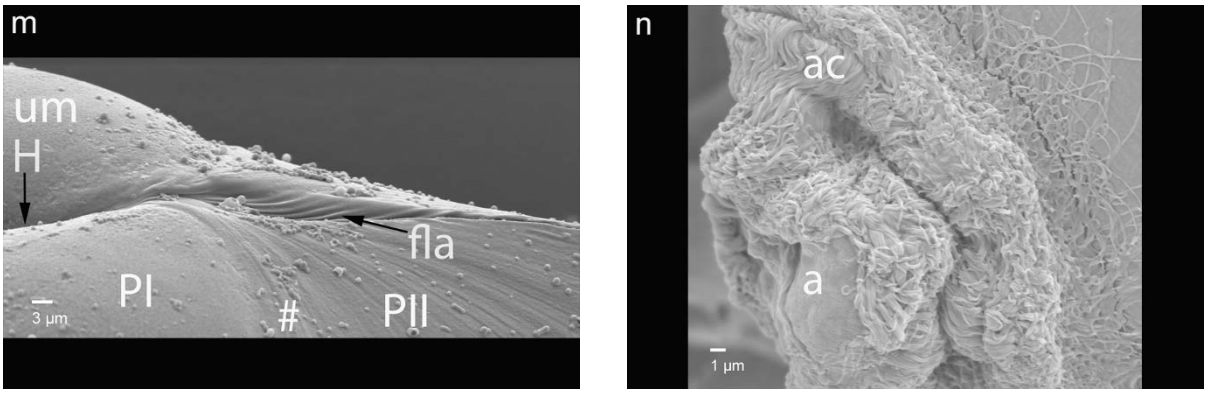
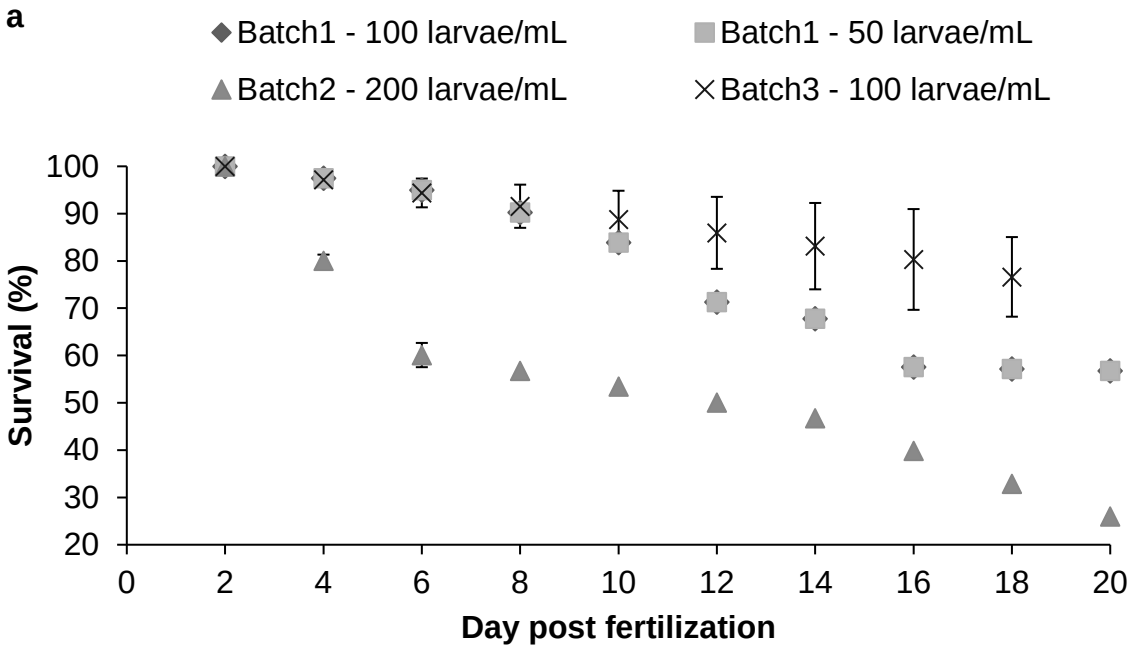


Figure 5.4 Standardized survival of *P. zelandica* larvae in three rearing batches a) from 2 dpf (based on initial stocking density) and b) from 6 dpf (based on 6 dpf stocking density) to the end of the larval rearing period.



b

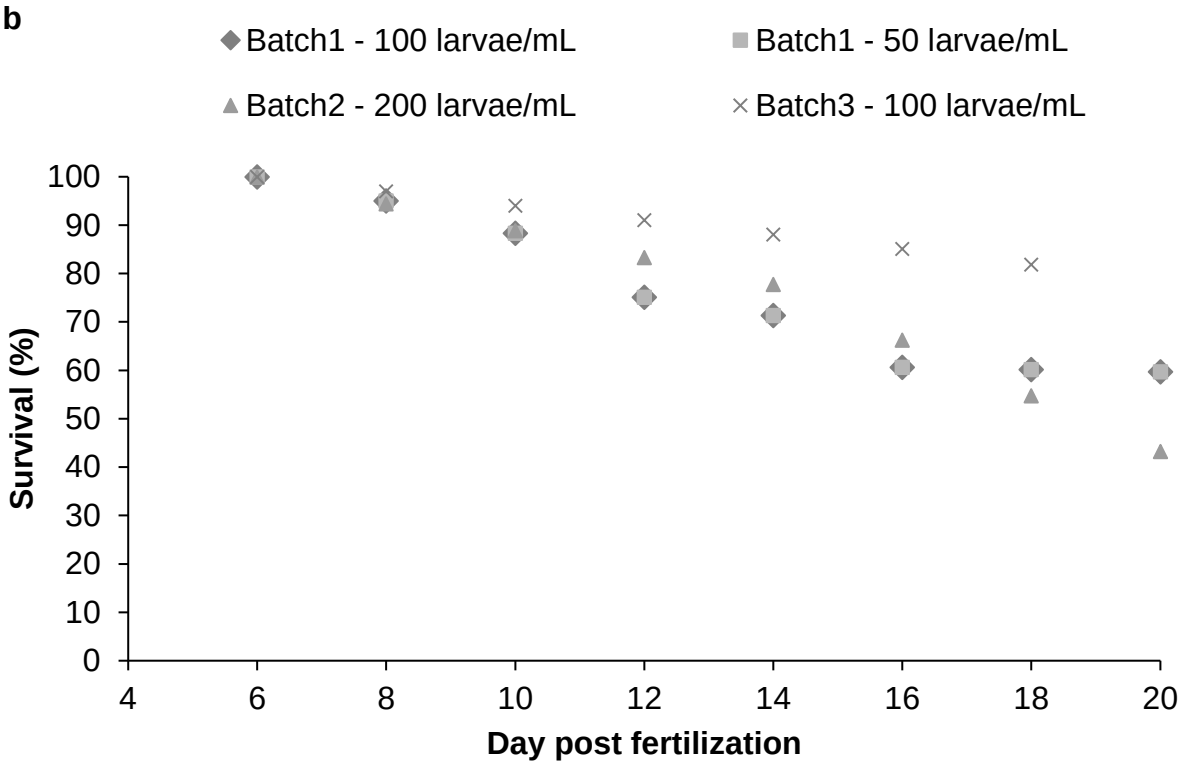

Batch1 - 100 larvae/mL
Batch1 - 50 larvae/mL
Batch2 - 200 larvae/mL
Batch3 - 100 larvae/mL
Survival (%)
100
90
80
70
60
50
40
30
20
10
0
4
6
8
10
12
14
16
18
20
Day post fertilization

Figure 5.5 Shell length of *P. zelandica* larvae in three batches assessed from first veliger to metamorphosis.

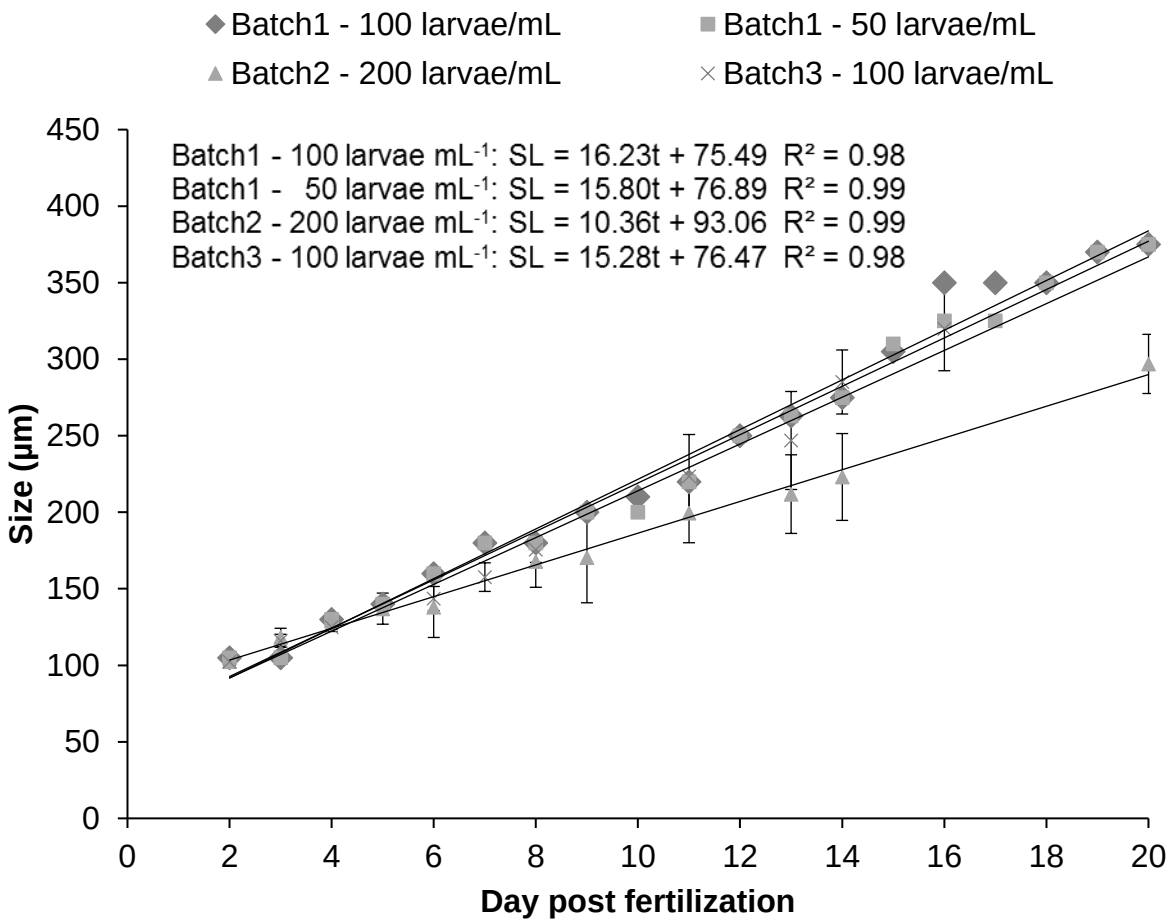


Figure 5.6 Specific growth rate of *P. zelandica* larvae shell length in three batches assessed from first veliger to metamorphosis.

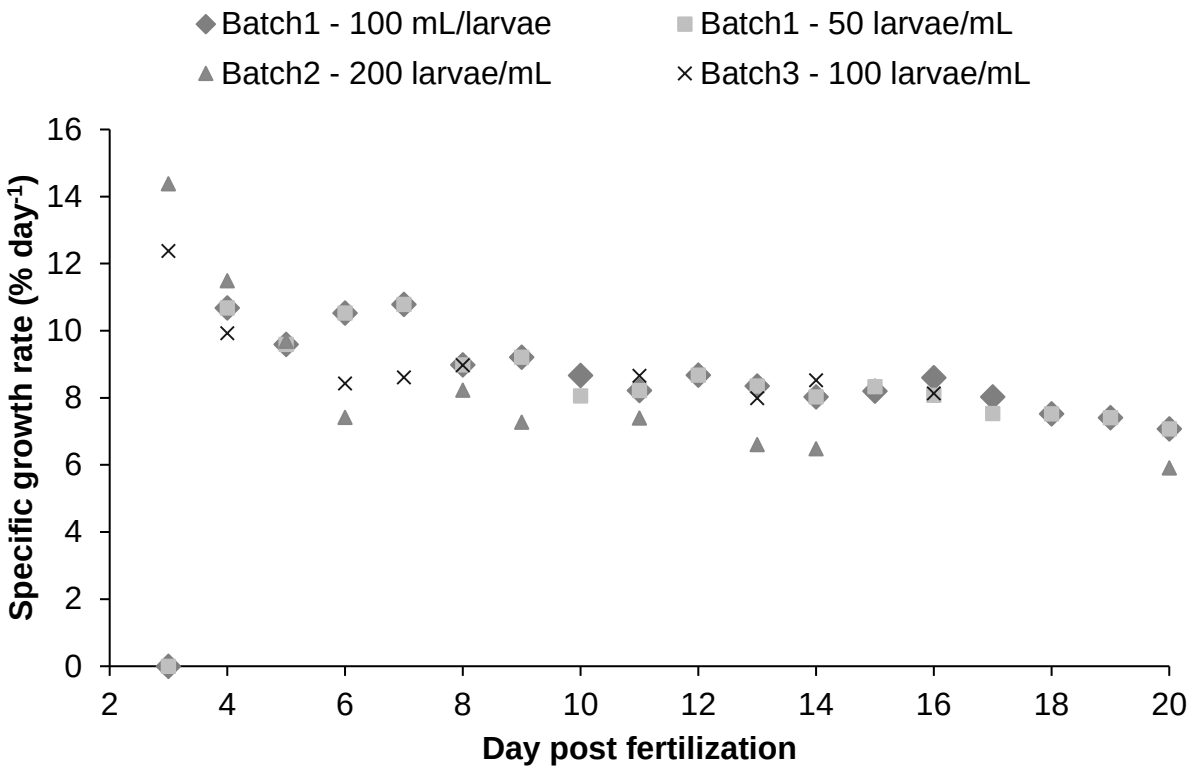


Figure 5.7 Gross shell growth rate (µm d^{-1}) of *P. zelandica* larvae in three batches assessed from first veliger to metamorphosis.

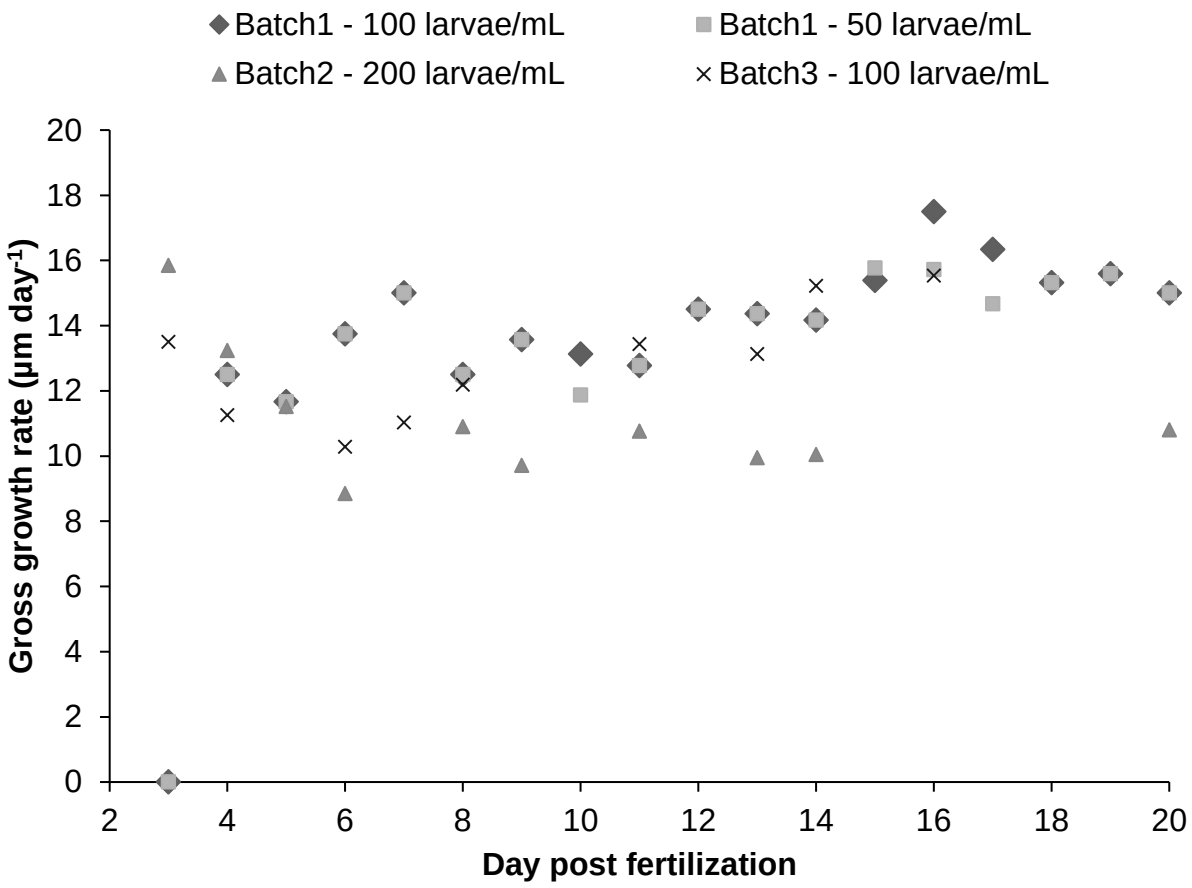
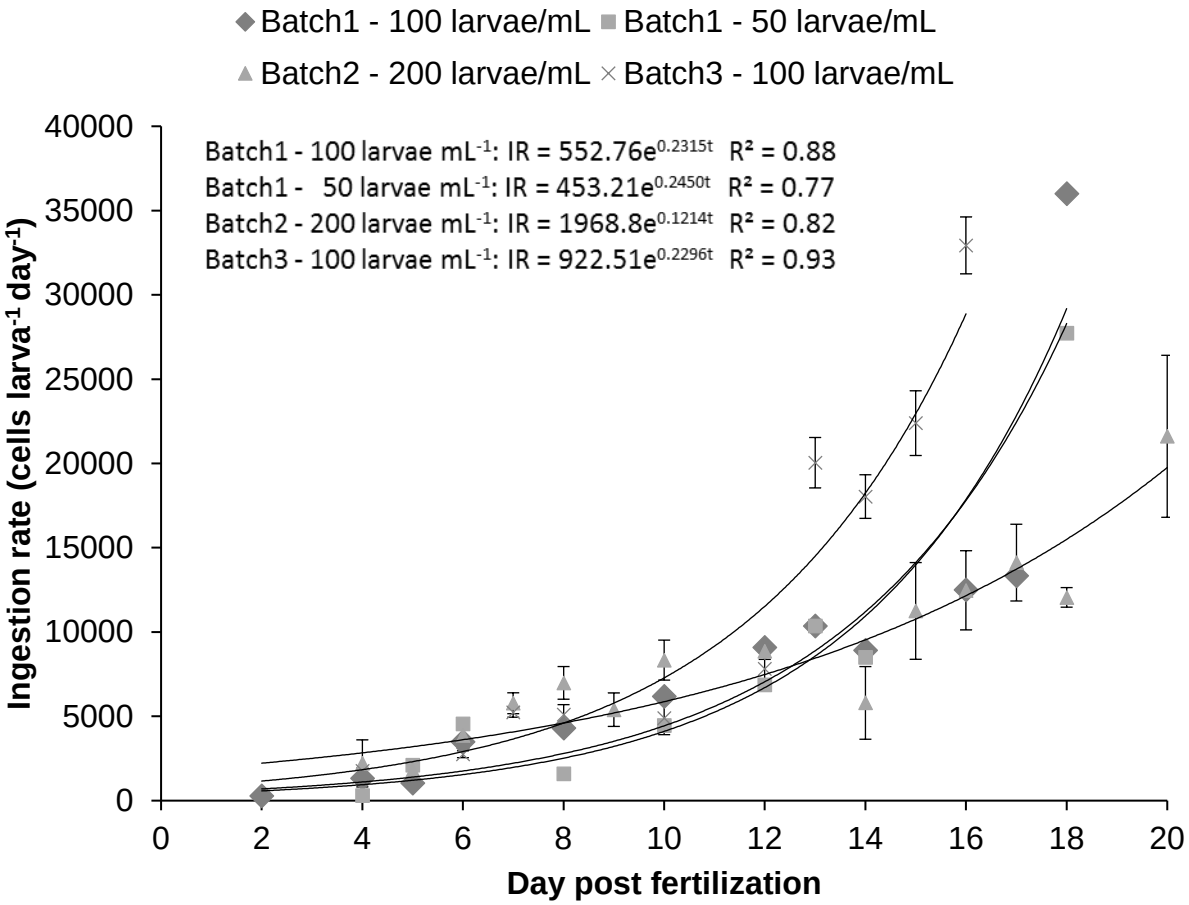
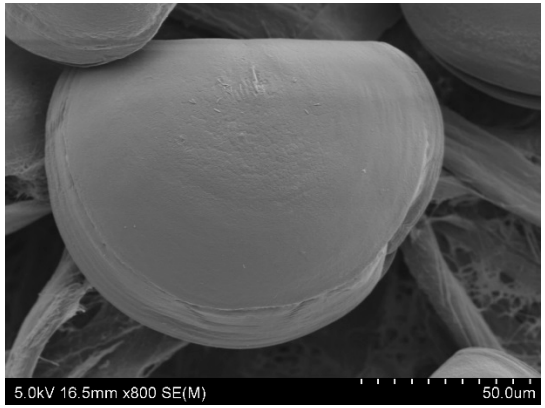


Figure 5.8 Ingestion rate of *P. zelandica* larvae in three batches assessed from first veliger to metamorphosis. Batch 1 received a feeding regime that maintained a residual cell concentration of 40,000 cells mL^{-1} until 10 dpf, then rising to 80,000 cells mL^{-1}; Batch 2 maintained 40,000 cells mL^{-1} and Batch 3 maintained 20,000 residual cells mL^{-1}.

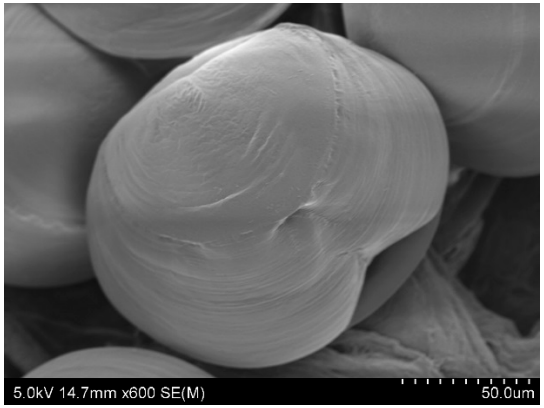


Supplementary 5.1 Figures of abnormal and normal shell of *P. zelandica* larvae. Scanning electron microscopy. **S1**, **S2**) Wave edged shell. **S3**) Complete closure valves. **S4**) Asymmetric valves. **S5**) Deformed shell. **S6**) Symmetric valves.

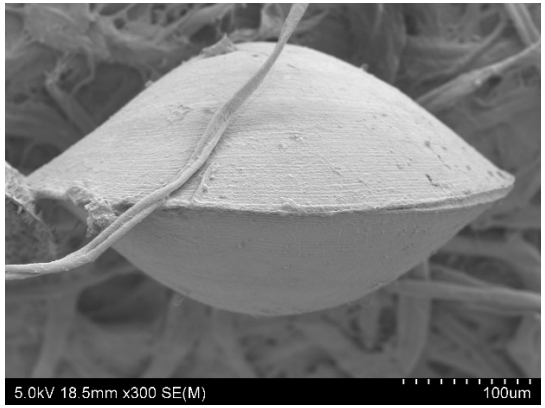
S1



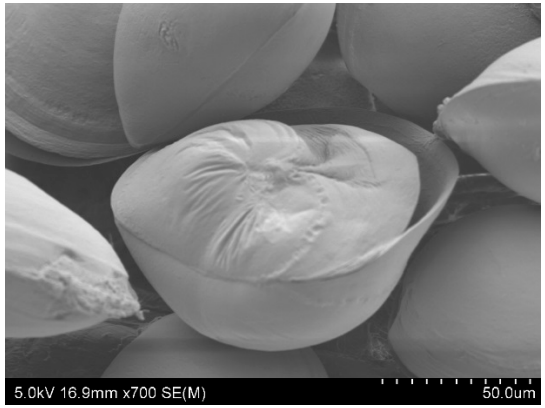
S2



S3

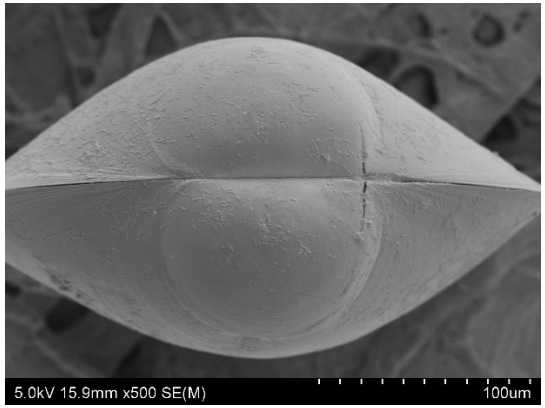


S4



S5

S6



Supplementary 5.2 Video of *P. zelandica* larva capturing algae. V1) pediveliger and V2) early spat.

V1	V2

CHAPTER 6 - Effect of neuroactive compounds on larval metamorphosis of New Zealand geoduck (*Panopea zelandica*)

This chapter was published as:

Le, D.V., Young, T., Alfaro, A.C., Watts, E., King, N. Effect of neuroactive compounds on larval metamorphosis of New Zealand geoduck (*Panopea zelandica*). Aquaculture Research.

Abstract

We present here the first laboratory study on the effects of pharmacologically active compounds on the larval metamorphosis of the New Zealand geoduck, *Panopea zelandica* (Quoy and Gaimard, 1835). Two batches of competent hatchery-reared larvae were exposed to acetylcholine chloride, epinephrine hydrochloride and excess potassium ions in the form of KCl and K_2SO_4. None of the tested chemicals increased the proportion of metamorphosed geoducks, and in some cases, the chemical caused significant mortality, despite having been used extensively with other species, such as mussels and oysters. This might indicate that geoduck larval physiology and development differs from other bivalves. Geoducks may have evolved distinct chemoreceptor patterns that facilitate metamorphosis under environmentally favourable conditions for subtidal soft sediment habitats suitable for burrowing. Thus, further research is needed to identify alternative cues (e.g. conspecific adults, sediment characteristics and surface biofilm) and understand their role in settlement and metamorphosis. This information will aid the design of reseeding methods and contribute to the development of reliable hatchery production of geoduck spat.

6.1 Introduction

Geoducks (*Panopea* spp.) are the largest burrowing clams in the world and are extremely long-lived. For example, *P. generosa* could live up to 168 years (Orensanz, Hand, Parma, & Valero 2004) and *P. zelandica* up to 86 years (Gribben & Creese 2005). However, despite their longevity and long-lasting fertility, their geographic distribution is limited (González-Peláez, Leyva-Valencia, Pérez-Valencia, & Lluch-Cota 2013; Gribben & Heasman 2015). Similar to other marine bivalves, geoducks produce planktonic larvae, which swim in the water column for a number of weeks before settling and metamorphosing into benthic juveniles. Various exogenous chemicals and other environmental stimuli typically activate larval settlement and metamorphosis (Hadfield & Paul 2001). Several physical, biological and chemical cues have been identified as inducers of larval settlement and/or metamorphosis across molluscan species (Hadfield & Paul 2001; Alfaro, Young, & Ganesan 2011). Such cues include substrate morphology (Hadfield & Paul 2001; Alfaro & Jeffs 2002), vibration (Rittschof, Forward, Cannon, Welch, McClary, Holm, Clare, Conova, McKelvey, Bryan, & van Dover 1998), water motion (Alfaro 2006), sound (Lillis, Eggleston, & Bohnenstiehl 2013), microbial biofilms (Ganesan, Alfaro, Brooks, & Higgins 2010; Tung & Alfaro 2011; Ganesan, Alfaro, Higgins, & Brooks 2012; Ganesan, Alfaro, Higgins, Duxbury, & Brooks 2012), the presence/absence of conspecifics and/or prey species (Hadfield & Paul 2001), and a range of natural or artificial chemicals (Steinberg, De Nys, & Kjelleberg 2002; Alfaro, Copp, Appleton, Kelly, & Jeffs 2006; Young, Alfaro, & Robertson 2011; Alfaro, Young, & Bowden 2014; Young, Alfaro, Sánchez-Lazo, & Robertson 2015). Specifically, chemical cues have been investigated for a range of bivalve species,

including oysters (Coon, Bonar, & Weiner 1985), mussels (Young, Alfaro, Sánchez-Lazo, et al. 2015), scallops (Mesías-Gansbiller, Bendimerad, Román, Pazos, Sánchez, & Pérez-Parallé 2008) and clams (García-Lavandeira, Silva, Abad, Pazos, Sánchez, & Luz Pérez-Parallé 2005). These chemical inducers enhanced significantly the proportion of larvae which metamorphosed into spat (i.e. >90% in oysters, Coon, Bonar, & Weiner 1986). However, none of these studies have tested the effects of the neuroactive compounds on geoducks.

Based on the distinct habitat requirements and patchy distribution of geoducks, it is likely that a specific set of cues is associated with settlement and metamorphosis of geoducks (Hadfield & Paul 2001). Indeed, it has been suggested that the patchy distributions of geoducks may be a result of chemical cues released from co-inhabiting polychaetes (*Spiochaetopterus costarum*, *Phyllochaetopterus prolifica* and *Dioptera ornate*), which may signal the presence of suitable recruitment habitats (Pease & Cooper 1988). Although these potential cues have not yet been identified, such compounds might bind to an assortment of epithelial-bound receptors and stimulate various endogenous biochemical processes (Hay 2009). Ultimately, these processes lead to dramatic physiological and morphological changes, which facilitate the transition of larvae from the pelagic to benthic phases of their life cycle.

Based on previous chemoreception studies with various bivalve species, there are several chemicals that could potentially induce geoduck larvae to settle and metamorphose, including γ-aminobutyric acid (GABA), acetylcholine, epinephrine and potassium ions (Alfaro et al. 2011; Young, Alfaro, Sánchez-Lazo, et al. 2015). At the same time, clear differences in induction abilities have been

observed among species, which leads to the conclusion that neuroactive inducers are highly species-specific, and these variations indicate the involvement of different mechanisms in the signal transduction responsible for triggering metamorphosis (Young et al. 2011).

GABA is produced by the decarboxylation of glutamic acid and acts as an inhibitory neurotransmitter (Kuffler, Nicholls, & Martin 1984), and it has been shown to induce either hyperpolarization of post-synaptic membranes by increasing the membrane permeability to chloride ions (Rodríguez, Ojeda, & Inestrosa 1993), or through depolarization of chloride flux (Kuffler et al. 1984). GABA is a powerful inducer of metamorphosis in the mussel *Mytilus galloprovincialis*, the clams *Venerupis pullastra* and *Ruditapes philippinarum*, the oyster *Ostrea edulis* (García-Lavandeira et al. 2005) and the scallop *Chlamys varia* (Mesías-Gansbiller et al. 2008). Acetylcholine (ester of acetic acid and choline) is a neurotransmitter in the peripheral nervous system (PNS) and central nervous system (CNS) (Martinez-Murillo, & Rodrigo 1994). Acetylcholine stimulates metamorphosis by acting on nicotinic- or muscarinic-type receptors, which may facilitate the downstream release of other neurotransmitters, such as GABA (López, Arce, Vicente, Oset-Gasque, & González 2001). Its stimulating effect has been observed in the oyster *Crassostrea gigas* (Beiras & Widdows 1995) and the clam *R. philippinarum* (Urrutia, Okamoto, & Fusetani 2004).

Epinephrine is a tyrosine derivative and catecholamine with various biological roles (e.g. acting as a hormone and neurotransmitter). Epinephrine stimulates adrenergic receptors to mediate metamorphosis (Qin, Huang, Chen, Zou, You, & Ke 2012) and is an active metamorphic inducer in the oysters *C. gigas*

(Coon et al. 1985), *O. edulis* (García-Lavandeira et al. 2005), *O. angasi* (O'Connor, Moltschaniwskyj, & O'Connor 2009), the mussels *M. galloprovincialis* (García-Lavandeira et al. 2005; Yang, Satuito, Bao, & Kitamura 2008; Yang, Li, Bao, Satuito, & Kitamura 2011), *M. coruscus* (Yang, Shen, Liang, Li, Bao, & Li 2013; Yang, Li, Liang, Li, Chen, Bao, & Li 2014) and the clams *V. pullastra* and *R. philippinarum* (García-Lavandeira et al. 2005).

Potassium is a universal regulator of ion gradients across cell membranes through which an electrical potential controls the conduction of electric impulses in nervous tissues (Barlow 1990). Potassium ions have been suggested to act on the PNS (Leitz & Klingmann 1990) and the CNS (Hadfield, Meleshkevitch, & Boudko 2000) via depolarization of excitable cells involved in the perception of inductive stimuli (Baloun & Morse 1984). The mechanism of potassium-induced larval settlement and metamorphosis likely relies more on voltage-gated potassium channels rather than inward rectifier potassium channels (Wang, Wu, Xu, Yu, Li, Li, Guo, & Wang 2015). Potassium ions triggered metamorphosis in the scallop *Argopecten purpuratus* (Martinez, Aguilera, & Campos 1999), the oyster *C. gigas* (Wang et al. 2015), and the mussels *M. galloprovincialis* (Yang et al. 2008) and *M. coruscus* (Yang et al. 2014).

The New Zealand geoduck clam (*P. zelandica*) has been identified as one of the emerging species to contribute to the target of NZ$1 billion export value within the New Zealand aquaculture sector by 2025 (Carter, 2012). However, in order for this species to be successfully cultivated, a reliable hatchery production of spat needs to be established, where quality and quantity can be modulated to fit the industry's demands. As a starting point, information is needed with regards to

critical stages in geoduck larval development, such as metamorphosis, and how these processes can be synchronized to achieve effective and efficient spat production. No study has examined those aspects so far, hence, in practice geoduck larvae are simply allowed to metamorphose with no special treatment (Feldman *et al.,* 2004), resulting in low spat yields in previous production runs (Le pers. obs.). Since the settlement process consists of anchoring, which appears to involve leverage and grip rather than adhesion between the foot and sediment particles, it is impossible to determine settled larvae quickly and objectively (Le pers. obs.). Thus, the aim of this research is to investigate the effect of commonly used compounds (i.e., acetylcholine, epinephrine, and potassium ions) on metamorphosis and mortality of *P. zelandica* larvae after short and long-term exposures.

6.2 Materials and Methods

6.2.1 Larval source

Adult *P. zelandica* from Bay of Plenty and Golden Bay areas were conditioned (details in Le, Alfaro & King, 2014) at the Cawthron Institute, Nelson, New Zealand. The Golden Bay broodstock were spawned in March 2014 while the Bay of Plenty broodstock were spawned in October 2014. Gametes were pooled and mixed from 5–10 adults to ensure genetic heterogeneity. Fertilized eggs were incubated in 1 µm filtered seawater (FSW) at 17–18°C and 35 ppt salinity. Once over 90% of organisms had reached the D-larval stage (2 days post fertilization), they were transferred to specialized 2.5 L flow-through tanks (CUDL system [see Ragg *et al.,* 2010]). Larvae were reared at 17–18°C with a mixed diet of *Chaetoceros calcitrans* and *Tisochrysis lutea* (formely known *Isochrysis affinis galbana* or *T-ISO*

clone; Bendif *et al.,* 2013), which was introduced continuously to allow *ad libitum* food supply. After 18–21 days post-fertilization, larvae had developed into pediveligers. At this stage, larvae tend to aggregate at the surface of the static water and the transparent statocysts become observable, indicating competency to settle and metamorphose. Pediveligers were screened on a 175 µm nylon mesh, and those which were retained were transferred to 1 L beakers. Only swimming larvae in the beakers were decanted to another beaker and adjusted to a density of 30–40 larvae mL^{-1} with FSW.

6.2.2 Treatment solutions

Stock solutions of KCl, K_2SO_4, acetylcholine chloride, and epinephrine hydrochloride (all chemicals were purchased from Sigma Aldrich, Sydney, Australia) were prepared at concentrations based on the effective ranges in the literature for clams, mussels, and oysters. The effect of potassium ions on larval metamorphosis was determined by two potassium-containing compounds (KCl and K_2SO_4) with different anionic compositions. The final K^+ exposure concentrations from these salts were 5, 10 and 20 mM. The final exposure concentrations of acetylcholine chloride and epinephrine hydrochloride were 10^{-3}, 10^{-4}, and 10^{-5} M.

6.2.3 Metamorphosis assays

Experiment 1

A completely randomized design was utilized to determine the effect of different concentrations of KCl, K_2SO_4, acetylcholine chloride, and epinephrine hydrochloride on metamorphosis and mortality of geoduck larvae for 3 and 24 h at 18°C. These compounds were prepared as 10 × chemical stock solutions in MilliQ sterile water immediately prior to animal exposures. Treatment assays consisted 8

ml FSW (0.45 µm), 1 mL larval solution (30–40 larvae), and 1 mL chemical stock solution following protocols in Coon *et al.* (1985) and Young *et al.* (2011) in which the 10% reduction in salinity had no effect on their experimental results. Control assays consisted of 8 mL FSW, 1 mL larval solution and 1 mL MilliQ sterile water. The pH of epinephrine solutions was reduced to 7.6 and not corrected following the effective protocol for oysters, as described in Coon *et al.* (1985).

For the 3 h exposures, to minimize handling stress, larvae were held in 100 µm mesh cups (diameter x height: 30 mm x 50 mm) which were placed in Petri dishes (diameter x height: 50 mm x 20.3 mm) containing the treatment solutions. After 3 h, larvae were rinsed, then placed in a tray containing FSW and fed *T. lutea* for further 21 h to avoid food deficiency following the protocol in O'Connor *et al.* (2009). The food deficiency at the metamorphosis stage has been shown to have a negative effect on metamorphosis of oyster larvae (Laing, 1995). In the 3 h exposure assay, four replicates were utilized for each treatment and the control. For the 24 h exposures, larvae were placed directly in the petri dishes containing the treatment solutions. Except for the substrate difference, larvae were treated the same for both 3 and 24 h exposures. There were 10 replicates for each treatment and the control for the 24 h exposures.

All larvae (~ 6400 individuals) were assessed for metamorphosis and mortality after 24 h from the commencement of assays under an inverted microscope at 100 × magnification. Larval metamorphosis is a definitive morphogenetic event, which includes loss of larva-specific organs (e.g., those used in swimming) and emergence of juvenile-specific structures (Hadfield & Paul, 2001). Hence, geoduck larvae which had completely lost their velum were

classified as metamorphosed. Empty shells and larvae which did not display any movement of velum, foot, and gut were classified as dead.

Experiment 2

A second experiment with the same completely randomized design, but different solution preparations was conducted with larvae stemming from Bay of Plenty broodstock. Instead of MilliQ sterile water, KCl, acetylcholine chloride, and epinephrine hydrochloride were directly dissolved in 0.45 µm filtered seawater to the desired concentration immediately prior to animal exposures. This modification was to avoid the effect of salinity reduction if there was any. In addition, the pH of the epinephrine solution was corrected to the pH 8.2 of FSW by adding sodium hydroxide to avoid the potential effect of pH reduction. Since the inductive effects of K^+ could be made with increased confidence to the cationic component of the compounds in the first experiment, we used only KCl in the second experiment.

For both 3 h and 24 h exposures, to minimize handling stress, larvae were kept in mesh cups which were placed in trays containing the treatment solutions at 18°C. After the exposure durations, larvae were rinsed and placed in a tray containing FSW and fed with *T. lutea*. There were 10 replicates for each treatment and the control. From the first experiment, it was observed that one of the indicative features of geoduck metamorphosis was the growth of new shell with developing spines after 48 h. Thus, for the second experiment, geoduck larvae were fixed with Davidson's solution after the assays and then assessed for metamorphosis and mortality in the following 24 h. All larvae (~ 6200 individuals) were assessed for metamorphosis and mortality. Larvae which displayed

developing spines were classified as metamorphosed and empty shells were classified as dead.

Percent metamorphosis was determined as the ratio of the number of metamorphosed larvae over the total number of animals in a Petri dish or in a mesh cup. Mortality was determined as the ratio of the number of dead larvae over the total amount of animals in a Petri dish or in a mesh cup.

6.2.4 Statistical analysis

Percent metamorphosis and mortality data were arcsine transformed prior to parametric statistical analysis to stabilize variances and normalize proportional data. Transformed data of metamorphosis and mortality of treatments and control were compared by one-way analysis of variance (ANOVA) with pair-wise Tukey's *post-hoc* tests with a significant level of 0.05. Means that are not significantly different from one another have the same letter above the bars in figures. Statistical analyses were conducted using the Minitab software version 17. All percentage data are expressed as mean ± SD.

6.3 Results

6.3.1 Experiment 1:

After both 3 h and 24 h exposures, none of the compounds tested resulted in enhanced metamorphosis compared to the controls, and greater mortalities were observed within all the treatments compared to the controls (Fig. 6.1 & 6.2).

The percent metamorphosis of the control after 3 h was significantly higher than that of the 20 mM KCl treatment and not significantly different from that of the other 11 treatments (Fig. 6.1a). However, the mortality of the control was not

significantly different from that of the 10^{-5} M acetylcholine, and 10^{-5} M and 10^{-4} M epinephrine and significantly lower than that of the other 9 treatments after 3 h (Fig. 6.1b).

In the 24 h exposure assay, the percentage of metamorphosed larvae of the control was significantly higher than that of 10^{-3} M acetylcholine and 10^{-3} M epinephrine treatments, but not significantly different from that of the other 10 treatments (Fig. 6.1c). In addition, the mortality in the control was not significantly different from that of 10^{-5} M epinephrine and significantly lower than that of the other 11 treatments after 24 h (Fig. 6.1d).

6.3.2 Experiment 2:

The same metamorphosis pattern was observed in the second experiment. The control was not significantly different in the percent metamorphosis and the mortality from all of 9 treatments after 3 h (Fig. 6.2a, b). In the 24 h exposure assay, the larval metamorphosis and mortality in the control were not significantly different from that of the acetylcholine, epinephrine, and KCl treatments, excluding the 10^{-3} M epinephrine treatment which was toxic (Fig. 6.2c, d).

6.4 Discussion

The objective of this study was to determine whether neuroactive compounds could induce metamorphosis of geoduck larvae, as is the case for many other bivalve species. Contrary to our expectations, our findings revealed that there was no positive effect of the tested compounds at studied concentrations on metamorphosis, and in some cases, the chemicals tested had high toxicity effects on geoduck larvae.

A range of neuroactive compounds and ions have been shown to induce metamorphosis in bivalves, such as GABA, epinephrine, acetylcholine, K^+ and some of these are regularly used in aquaculture production of oysters (Helm, Bourne & Lovatelli, 2004). However, it also has been recognized that a chemical which may stimulate settlement and/or metamorphosis in larvae of one bivalve species may not be effective on larvae of another species (García-Lavandeira *et al.,* 2005; O'Connor *et al.,* 2009; Young, Alfaro, Sánchez-Lazo *et al.,* 2015). A summary of the effects of acetylcholine, epinephrine and potassium ion at different concentrations on the metamorphosis in larvae of different species is presented in Table 6.1.

Unlike other bivalve species which respond positively to at least one of the artificial neuroactive compounds tested (Table 6.1), geoduck larvae had no positive response to any of these chemicals at the concentrations used in this study (and in one single experiment). It is possible that a different set of chemical inducers (e.g., sediment surface biofilms, conspecific adults) is needed for these subtidal clams. For instance, a dialysate (containing substances which have molecular weights < 1.2 kDa) of autoclaved native sediment was shown to enhance metamorphosis of geoduck *P. generosa* larvae (King, 1986). Multi-species bacterial biofilms from natural habitats have been reported to induce settlement and/or metamorphosis in several bivalve species (e.g., oysters, Tamburri *et al.,* 2008; Zhao, Zhang & Qian, 2003, and mussels, Ganesan *et al.,* 2012b; Wang *et al.,* 2012). If surface biofilms are shown to be inducers for *P. zelandica* in the future studies, then variations in these biofilms may be responsible for the observed patchy distribution of *P. zelandica*, and their absence in habitats that appear to be suitable for them

(Goodwin & Pease, 1991). Such species-specific adaptations to micro-environmental conditions have been reported for other species and are thought to be crucial for survival and growth. For example, oyster larvae settle and metamorphose preferentially on conspecific adult shells, which contain glycoprotein-inducing substances within the organic shell matrix (Vasquez *et al.,* 2013, 2014). Similarly, abalone larvae may settle as a response to mucus trails of conspecific juveniles, which may signal suitable settlement substrates (Gallardo & Buen, 2003; Laimek *et al.,* 2008).

It is clear that further studies are needed to investigate the induction of settlement and metamorphosis in *P. zelandica*. The non-results in these trials suggest that inducers used effectively in other bivalve species may not be transferrable to *P. zelandica*. Therefore, an approach based on gaining a better understanding of the likely triggers and metamorphosis process in *P. zelandica* may be appropriate. Such studies with potential inducers (i.e. conspecific adults and bacterial biofilms) may involve 'omic' approaches, which are showing great promise to elucidate subtle physiological responses in a range of marine species. For example, comprehensive expression patterns of receptor-encoding genes, various proteins, and metabolites during larval development have been demonstrated for some bivalve species, such as oysters (*C. angulata*, Qin *et al.,* 2012; and *C. gigas*, Huan *et al.,* 2012a; Huan, Wang & Liu, 2015), clams (*Meretrix meretrix*, Huan, Wang & Liu, 2012) and mussels (*Perna canaliculus*, Young, Alfaro & Villas-Bôas, 2015a, b), and may prove to be useful to elucidate the unusual early life history of *P. zelandica*.

While the lack of a response to chemical inducers tested in this study was unexpected, the results are consistent with observations of spontaneous metamorphosis in hatchery larval rearing tanks (Feldman *et al.,* 2004). Newly metamorphosed geoduck spat appeared to remain in suspension, with some attached spat on the tank wall and air/feeding rods in the larval rearing system for at least 24 h post-metamorphosis. It is possible that this species adopts the 'variable retention' strategy (Bishop *et al.,* 2006) where metamorphosis is effectively decoupled from settlement, with competent larvae metamorphosing in the water column, and then sinking to the benthos as fully developed spat to identify suitable sites for settlement. The capacity to skip the settlement process, then metamorphose in the water column is supported by the byssal secretion behavior which only occurs at the late metamorphosis (King, 1986).

In summary, the metamorphosis and mortality percentages of *P. zelandica* larvae were highest in the control. *P. zelandica* larvae were not induced to metamorphose with 10^{-5}, 10^{-4}, and 10^{-3} M acetylcholine and epinephrine, and 5, 10, and 20 mM potassium ions during 3 h or 24 h exposure time. Furthermore, the 24 h exposures resulted in significant mortalities at 10^{-3} M epinephrine. It would be helpful in the future to test for the lower concentrations to avoid toxic effects and add more confirmation of results of this study. Further research with other potential inducers, such as conspecific adults and bacterial biofilms, in combination with 'omics' approaches may help elucidate the potential induction and mechanism responsible for geoduck larval settlement and metamorphosis. Such information would improve our standing of the geoduck recruitment process, and would be

invaluable for further development of geoduck hatchery production of spat for wild re-seeding and aquaculture purposes.

6.5 References

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Table 6.1 Effect of chemical cues and time exposure on metamorphosis of bivalve larvae

Group	Species	Acetylcholine			Epinephrine			K^+			Reference
		10^{-5} M	10^{-4} M	10^{-3} M	10^{-5} M	10^{-4} M	10^{-3} M	5 mM	10 mM	20 mM	
Clams	*Cyrtopleura costata*	- ~	- ~	- ~	+ ↓	-/+ ↑	+ ⊗	nd	-	nd	Gustafson *et al.,* 1991
	Ruditapes philippinarum	+ ~	+ ~	nd	-/+ ~	-/+ ~	nd	nd	nd	nd	Urrutia *et al.,* 2004
	R. philippinarum	nd	nd	nd	+ ↑	+ ↑	nd	nd	nd	nd	Lu *et al.,* 2006
	R. philippinarum	nd	nd	nd	- ↑	- ~	nd	nd	nd	nd	García-Lavandeira *et al.,* 2005
	Venerupis pullastra	nd	nd	nd	+ ↑	+ ↑	nd	nd	nd	nd	García-Lavandeira *et al.,* 2005
	Panopea zelandica	- ~	- ~	- ~	- ~	- ~	- ~	- ~	- ~	- ~	Present study
Oysters	*Crassotrea gigas*	-	-/+	nd	+	+ ↑	⊗	nd	nd	nd	Coon *et al.,* 1985
	C. gigas	-	-/+	-	-/+	+ ~	-	nd	nd	-	Beiras & Widdows, 1995
	C. virginica	nd	nd	nd	-/+	+ ↑	-	nd	nd	nd	Coon *et al.,* 1986
	Ostrea angasi	nd	nd	nd	-	+ ↓	- ↓	nd	nd	nd	O'Connor *et al.,* 2009
Mussels	*Mytilus galloprovincialis*	nd	nd	nd	+ ↑	- ↑	nd	nd	nd	nd	García-Lavandeira *et al.,* 2005
	M. galloprovincialis	nd	nd	nd	+ ~	+ ↓	nd	nd	- ~	nd	Yang *et al.,* 2008
	M. coruscus	-/+	-/+	nd	-/+ ↓	+ ↓	nd	nd	-/+	nd	Wang *et al.,* 2012
Scallops	*Argopecten purpuratus*	nd	nd	nd	+ ↑	- ~	nd	- ~	+ ↑	- ~	Martinez *et al.,* 1999

Notation for the effect of chemicals: - = no effect, -/+ = low effect, + = positive effect, ⊗ = toxic effect, nd = no data. Notation for the effect of long term exposure over short term exposure: ~ = same effect, ↑ = increased effect, ↓ = decreased effect, ⊗ = toxic effect.

Figure 6.1 Mean (±SD) percent metamorphosis and mortality of geoduck larvae after 3 and 24 h in seawater (controls) and exposure to different concentrations of acetylcholine, epinephrine, KCl and K_2SO_4 in experiment 1. Different letters above the bars indicate pair-wise differences between treatments ($P<0.05$).

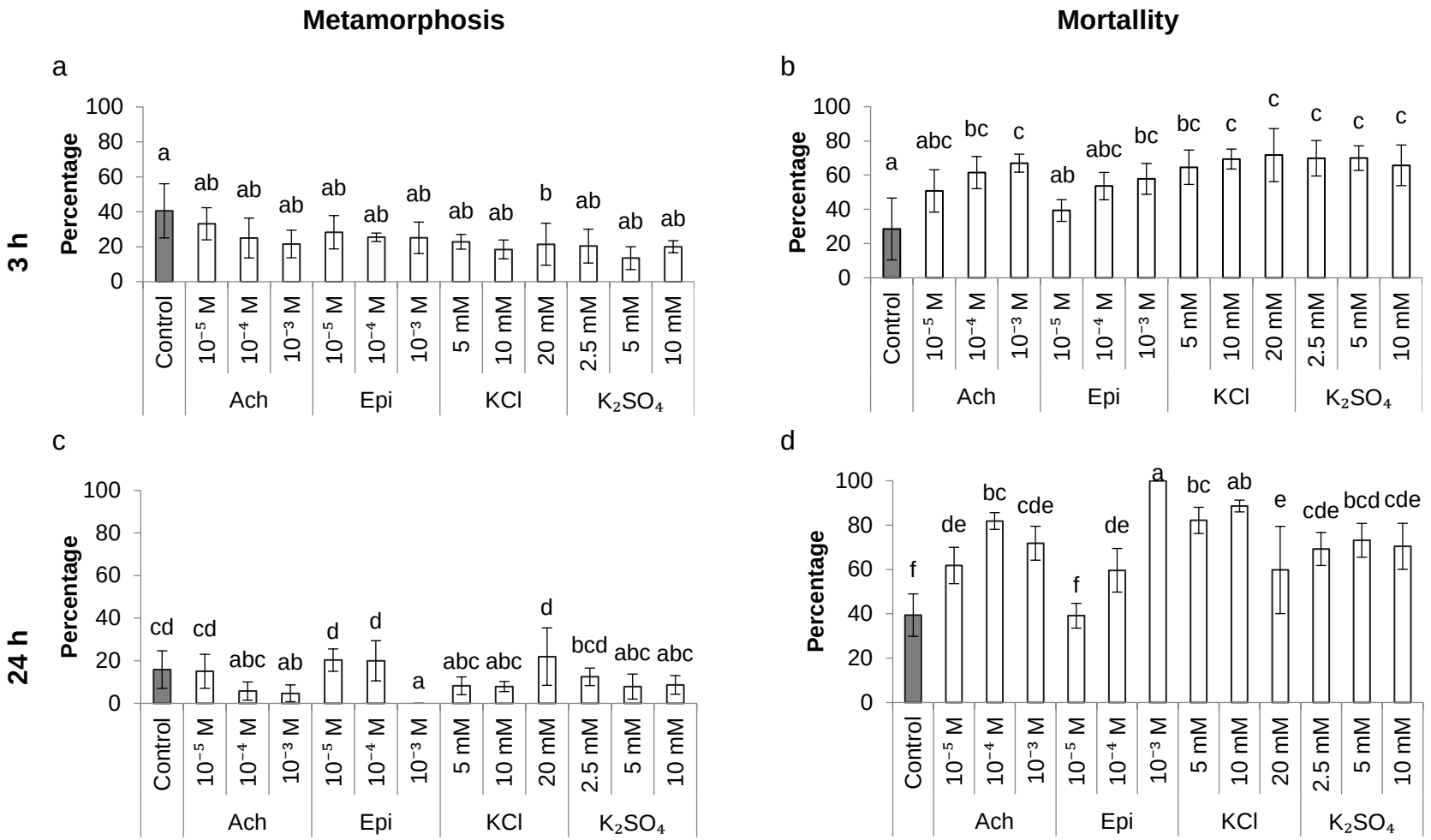
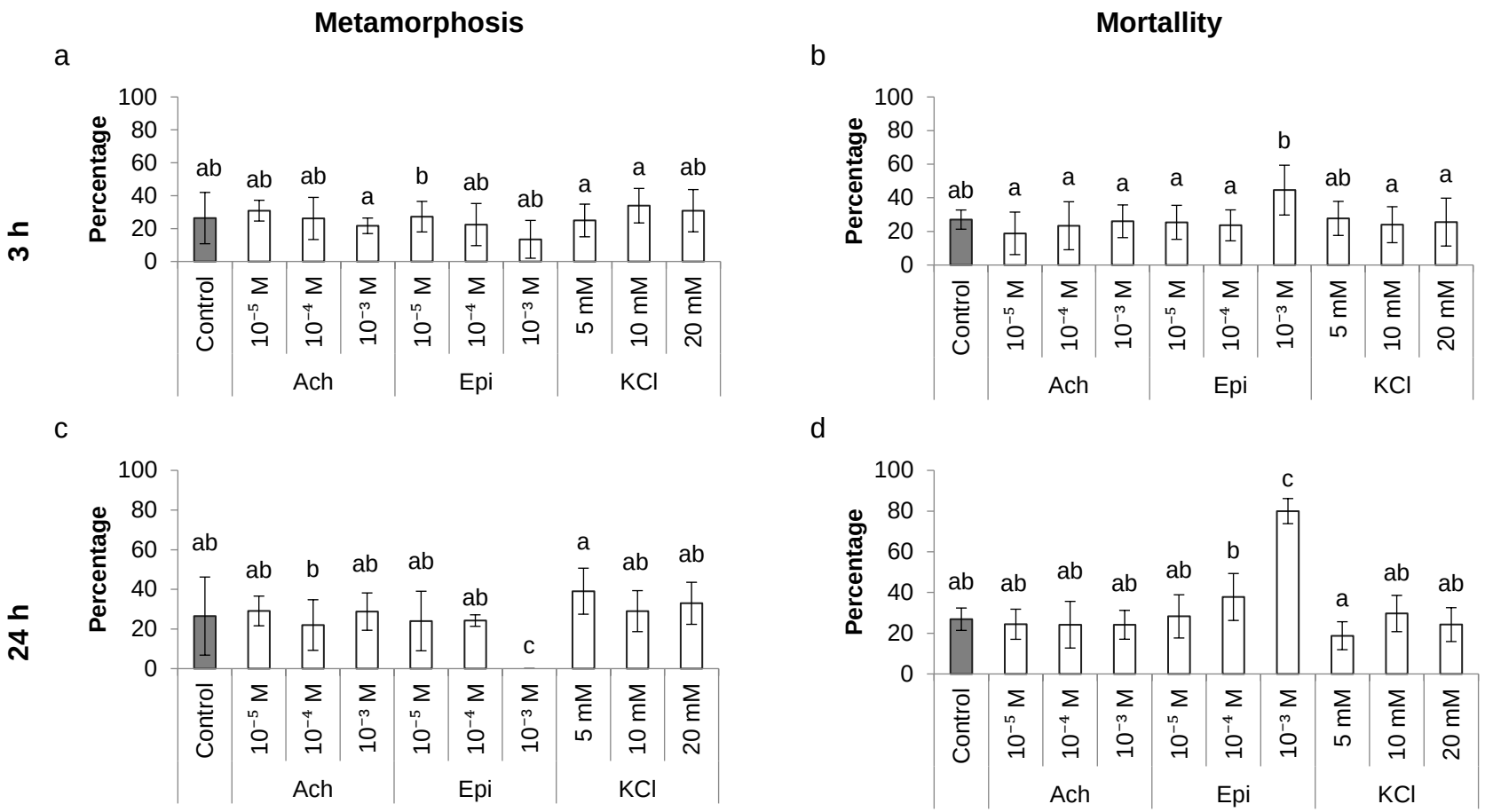

Metamorphosis
Mortallity
a
b
c
d
3 h
24 h
Percentage
Control
10⁻⁵ M
10⁻⁴ M
10⁻³ M
5 mM
10 mM
20 mM
2.5 mM
Ach
Epi
KCl
K₂SO₄

Figure 6.2 Mean (±SD) percent metamorphosis and mortality of geoduck larvae after 3 and 24 h in seawater (controls) and exposure to different concentrations of acetylcholine, epinephrine, and KCl in experiment 2. Different letters above the bars indicate pair-wise differences between treatments (P<0.05).


Metamorphosis
Mortallity
a
b
c
d
3 h
24 h
Percentage
100
80
60
40
20
0
ab
a
b
c
Control
10⁻⁵ M
10⁻⁴ M
10⁻³ M
5 mM
10 mM
20 mM
Ach
Epi
KCl

CHAPTER 7 – Allometric scaling of physiological rates in the New Zealand geoduck clam, *Panopea zelandica*

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Abstract

The New Zealand geoduck clam *Panopea zelandica* (Quoy and Gaimard, 1835) is among the largest burrowing clams worldwide. However, the effects of size scaling on physiology have not been studied for any geoduck species. In this study, we determined the allometric coefficients of respiration rate (RR) and clearance rate (CR) for *P. zelandica*. The allometric coefficients (*β*) of RR and CR in *P. zelandica* were 0.73 ± 0.03 and 0.62 ± 0.07, respectively. These coefficients are eligible for the mass exponent correction in physiological studies for *P. zelandica*.

7.1 Introduction

In governing animal function, physical laws are as important as biochemical processes; they determine rates of diffusion and heat transfer, transfer of force and momentum, the strength of structures, and the dynamics of locomotion (Schmidt-Nielsen, 1975). Consequently, body size influences virtually every structural and functional trait of an organism (Karasov and Martinez del Rio, 2007). Indeed, the physiological functions of living creatures, including bivalves, were proposed to change with size following a power law: $Y = aX^b$ (Schmidt-Nielsen, 1984). Y represents a physiological process (e.g., metabolic rate, clearance rate), X is typically a measure of mass, *a* is a conditional factor, and *b* is the allometric factor. The *a* constant denotes the level of physiological rate of individuals per unit body weight, which can be used to compare physiological rates among populations or species (Schmidt-Neilsen, 1975). For example, the mean *a* value of respiration rate for bivalves is lower than that of grazing gastropods, suggesting that the energetic cost of filtration by bivalves is systematically lower than that of grazing in gastropods (Bayne and Newell, 1983). The constant *b* denotes the rate change of physiological rate with changing body mass. Hence, this value is used to compare the scaling relationship between physiological rates and size among populations or species (Schmidt-Neilsen, 1975). Since physiological rates are generally strongly dependent on a non-linear relationship with body weight, the *b* exponent is also used for a weight correction, allowing comparisons between animals of different sizes (Bayne and Newell, 1983). Due to numerous exogenous and endogenous factors, the proportional allocation of energy to somatic growth or reproduction varies extensively among species, and even among populations of the same

species (Gosling, 2003). As a result, there is no common allometric factor for physiological rate in bivalves (Bayne and Newell, 1983, Riisgård, 1998, McDonald et al., 2006 and Vladimirova et al., 2003). Thus, it is recommended that, as a minimum, the allometric values of respiration and clearance rates are determined for the targeted experimental organism (Bayne and Newell, 1983 and Riisgård, 1998). These allometric relationships are necessary to accurately estimate physiological measurements, such as the growth efficiency during ontogeny, and therefore contribute to understanding the production cycle and informing husbandry practices.

Geoducks are one of the largest burrowing clams with the longest siphons, which are 4-5 times longer than their shell length. The Pacific geoduck, *P. generosa*, attains a shell length of over 212 mm and a live weight of 3.25 kg (Goodwin and Pease, 1989) and the Cortez geoduck, *P. globosa*, grows up to 205 mm in shell length and 2.8 kg in live weight (González-Peláez et al., 2015). The Japanese geoduck, *P. japonica*, attains 107 mm in shell length and 550 g in live weight (Lee et al., 1998), while the largest reported size of Argentine geoduck, *P. abbreviata*, is 126 mm in shell length and 651 g in live weight (Morsan et al., 2010). In addition, the Mediterranean geoduck, *P. glycimeris*, is reported to have a shell length of 305 mm (Scotti et al., 2011) and the New Zealand geoduck, *P. zelandica*, can achieve 135 mm in shell length and ~ 1 kg in live weight (Breen et al., 1991).

To our knowledge, the allometric factors of respiration rate and clearance rate have not been established for any geoduck clam, which is considered as one of the world's largest burrowing clams. They possess long, conjoined inhalant-exhalant siphons, which are 4 – 5 times longer than shell length, hence; can dig

down up to 1 m below the sediment surface (Goodwin and Pease, 1989). The sedentary behaviour, within a relatively deep and stable sub-seafloor environment, may contribute to their long life spans, which have been recorded to be as long as 168 y for *P. generosa* (Orensanz et al., 2004) and 86 y for *P. zelandica* (Gribben and Creese, 2005). Furthermore, geoduck shells are not able to close, and their large mantles cannot be fully retracted inside the valves. Based on these unique life strategies, it is expected that they also may have distinctive physiological energetics, especially in relation to size and to growth effects. Hence, there is a clear need to determine the ontogenetic allometric relationships of physiological rates for geoducks. Thus, the aim of this study was to investigate the dependence of respiration rate and clearance rate on size in *P. zelandica*.

7.2 Materials and Methods

7.2.1 Animals

Large geoducks (89 - 126 mm maximum shell length) were collected from Golden Bay, South Island, New Zealand. Since it is hard to locate and collect smaller geoducks from the wild, medium sized (40 - 72 mm) and small geoducks (14 – 24 mm shell length) were sourced from stock spawned and grown at the Cawthron Institute, Nelson, from Golden Bay broodstock (Le et al., 2014). To determine the relationship of physiological traits and size, 5 large, 5 medium, and 5 small geoducks were used. Before physiological measurements were made, each geoduck was individually buried in sand (< 1 mm in grain size) in 50 mL Falcon™ tubes (for small geoducks), acrylic plastic tubes (~ 1270 mL; for medium geoducks), or PVC tubes (~ 7000 mL; for large geoducks). In this manner, the geoducks could be moved along with the tubes containing sand with minimal

handling stress. The sand was necessary to avoid added stress and extra energy expenditure by the adductor muscles that keep the valves from excessive gaping. All experimental tubes were kept vertical. All geoducks were conditioned in flow-through 1 µm filtered seawater at 15°C and a salinity of 35, and fed *Tisochrysis lutea* (formerly known as *Isochrysis affinis galbana or T-ISO* clone; Bendif et al. 2013, 2014*)* at 30,000 cells mL^{-1} for two weeks before measurements commenced.

7.2.2 Physiological measurements

Algal consumption

After two weeks of acclimation to the conditions above, 15 geoducks (buried in sand within tubes) were placed vertical in ~ 300 mL (for small geoducks) and ~ 8 L (for medium and large geoducks) individual flow-through tanks (Fig. 1a). Additionally, one tube (with sand but no geoduck) for each geoduck size class was put in an additional tank to serve as a blank control.

The flow-through method (Bayne et al., 1976) was applied to determine the rate of algal consumption for all geoducks. Geoducks were fed *T. lutea* at 30,000 cells mL^{-1} in 1-µm filtered seawater at 15°C. The algal concentration of the inflow and outflow of each tank was measured three times every 24 h over a 4 day period, using a Coulter Counter (Multisizer 4, Beckman Coulter). The flow rate of each tank was measured and adjusted three times a day so that only 15 – 25% of the algae were cleared by the geoducks. This amount of reduction was assumed to ensure reliability of measurements while allowing flow rates sufficient to prevent water re-filtration by the animals within the tanks (Crisp, 1971).

Oxygen consumption

Respirometers (660 ml volume) for small geoducks were made of acrylic plastic cylinders (60 mm Ø, 5 mm thick) with two gasket-sealed Perspex lids (10 mm thick) secured with threaded bolts and wingnuts (Fig. 1b). Each respirometer had a water inlet (lower lid) and outlet (upper lid). Small geoduck tubes were suspended inside the respirometers in which water was mixed by a magnetic flea (Fig. 1b). The acrylic plastic tubes (60 mm Ø, 5 mm thick) housing the medium geoducks and the PVC tubes (100 mm Ø, 10 mm thick) holding the large geoducks were converted into respirometers by sealing with two Perspex lids (Fig. 1c). Both the inlet and outlet were located on the upper lid. Additionally, one respirometer (with sand but no geoduck) of corresponding size was used as a blank control.

Routine oxygen consumption was measured following the flow-through method (Bayne et al., 1976) under the same temperature and feeding conditions as for the algal consumption measurements above. The oxygen consumption of individual geoducks was measured 1 h after placing animals in the respirometers, by which time the animals appeared to settle and open siphons' holes. The oxygen concentration at the outflow of each experimental chamber and the blank control was measured once every 9 h for 30 min (2 sec/recording), so that the reported values were an average of 3 measurements (i.e., an average of 900 recordings) over 27 h. The oxygen level was measured using a fibre-optic sensor (FOXY-R, Ocean Optics, Dunedin, FL) which was kept inside a custom-made water housing maintained at 15°C, and was connected to a phase measurement system (NeoFox®, Ocean Optics). This phase measurement system was connected to a computer using NeoFox software to record the oxygen values. The

oxygen sensor was initially calibrated with 100 and 0% air saturated water. The flow rates were measured and adjusted to achieve 10 - 20% difference in the oxygen level between the blank control and experimental respirometers, thus avoiding hypoxia and ensuring reliability of measurements. All respirometers were immersed in water baths to maintain a constant temperature of 15°C throughout the experiments.

7.2.3 Physiological rate calculation

The clearance rate was determined based on the algal consumption measurements following the equation (after Crisp, 1971):

$$CR = FR \times (C_{in} - C_{out})/C_{in}$$

where, CR is the clearance rate (L h^{-1}), FR is the flow rate through the chamber (L h^{-1}), C_{in} and C_{out} (cells mL^{-1}) are the algal concentrations of the inflow and outflow, respectively.

The respiration rate was determined based on the oxygen consumption measurements following the equation:

$$RR = FR \times (O_{in} - O_{out})$$

where, RR is the respiration rate (mg O_2 h^{-1}), FR is the flow rate (L h^{-1}) and O_{in} and O_{out} (mg O_2 L^{-1}) are the oxygen concentrations of the outflow of the control chamber and the experimental chambers, respectively.

7.2.4 Physical measurements

The shell lengths of 5 small and 5 medium geoducks were measured with digital callipers (± 0.01 mm). The tissue samples were dried at 104°C for 24 h and

weighed (± 0.0001 g; Mettler-Toledo balance; Columbus, OH). Since large geoducks were precious broodstock and were too valuable to sacrifice, their tissue dry weights were estimated based on the tissue dry weight - shell length relationship:

$Tissue\ Dry\ Weight\ =\ 0.000019\ \times\ Shell\ Length^{3.1243}$ (P = 0.001, R^2 = 0.99). The relationship between tissue dry weight and shell length of *P. zelandica* was determined using 18 large, 69 medium, and 35 small geoducks.

7.2.5 Data processing and statistical analyses

The relationships between physiological rates (clearance and respiration) and body size (tissue dry weight and shell length) were expressed according to the equations:

For tissue dry weight: $Y = \alpha \times (Tissue\ DW)^{\beta}$

and for shell length: $Y = \alpha \times SL^{\beta}$

where, Y is the respiration rate (mg O_2 h^{-1}) or clearance rate (L h^{-1}), Tissue DW is the tissue dry weight, SL is the shell length, and α and β are regression coefficients.

These equations were log-transformed following the formulas:

For tissue dry weight: $\log(Y) = \log(\alpha) + \beta \times \log(Tissue\ DW)$

and for shell length: $\log(Y)\ =\ \log(\alpha)\ +\ \beta\ \times\ \log(SL)$

The coefficients α and β were estimated by a linear regression analysis (least squares method). A t-test was applied to test whether β values were isometric (H_0:

β = 1) (Sokal and Rohlf, 1995). All statistical tests were evaluated at the significant level of α = 0.05 using Minitab v.17 statistical software.

7.3 Results

The regression analysis of log-transformed data indicated that clearance and respiration rates of *P. zelandica* were highly dependent upon size (*P* = 0.001, Table 7.1, Fig. 7.2). All coefficients of the physiological rates-size relationships were statistically significant (*P* = 0.001, Table 7.1).

The relationship between clearance rate or respiration rate and tissue dry weight were defined by the equations:

$$CR = 0.457 \pm 0.091 \times Tissue\ DW^{0.616 \pm 0.068}$$ (Fig. 7.2a, R^2 = 0.86)

$$RR = 0.336 \pm 0.025 \times Tissue\ DW^{0.733 \pm 0.027}$$ (Fig. 7.2b, R^2 = 0.98)

The corresponding relationships with shell length were:

$$CR = 0.00064 \pm 0.00101 \times SL^{1.890 \pm 0.233}$$ (Fig. 7.2c, R^2 = 0.84)

$$RR = 0.00012 \pm 0.00007 \times SL^{2.274 \pm 0.109}$$ (Fig. 7.2d, R^2 = 0.97)

7.4 Discussion

Clearance and respiration rates for *P. zelandica* showed a negative allometric relationship with tissue dry weight, as the scaling coefficients, β, were significantly less than 1 (Fig. 7.4). In the present study β = 0.62 for clearance rate, and β = 0.73 for respiration rate. Compared to other burrowing bivalves, the allometric coefficient of clearance rate of *P. zelandica* (0.62) is higher than that in *Leukoma staminea* and *Saxidomus giganteus* (0.517 and 0.430, respectively; Bernard, 1983) and *Cerastoderma edule* (0.56; Ibarrola et al., 2008), but lower than that of *Mya*

arenaria (0.71; Riisgård and Seerup, 2003). In addition, the allometric coefficient of respiration rate in *P. zelandica* (0.73) is higher than that of *Arctica islandica* (0.422; Taylor and Brand, 1975), *S. giganteus* (0.65; Bernard, 1983), *Cerastoderma edule* (0.62; Ibarrola et al., 2008), and *Ruditapes decussatu*s (0.707; Urrutia et al., 1999), but lower than that in *Clinocardium nuttallii* and *L. staminea* (0.78 and 0.83, respectively; Bernard, 1983), and *C. edule* (0.77; Smaal et al., 1997).

The allometric coefficients (*β)* of respiration rate and clearance rate in bivalves are generally considered to be species-specific (Bayne and Newell, 1983, Riisgård, 1998, Vladimirova et al., 2003 and Ibarrola et al., 2008). In addition, Ibarrola et al. (2008) revealed that these coefficients were not dependant on seasons in *C. edule*. Thus, the allometric coefficients of respiration rate and clearance rate of geoducks determined in the present study could reasonably be used to correct for the mass difference in future physiological studies of *P. zelandica*. Indeed, these coefficients were applied for both juvenile and adult geoducks in Le et al. (2016) so that their physiological rates were quantified correctly.

In summary, the physiological rates of *P. zelandica* have negative allometric relationships with size, with allometric coefficients of 0.62 for clearance rate and 0.73 for respiration rate.

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Table 7.1 ANOVA results of regression analysis between clearance rate, respiration rate and tissue dry weight (DW) or shell length (SL) after log transformation.

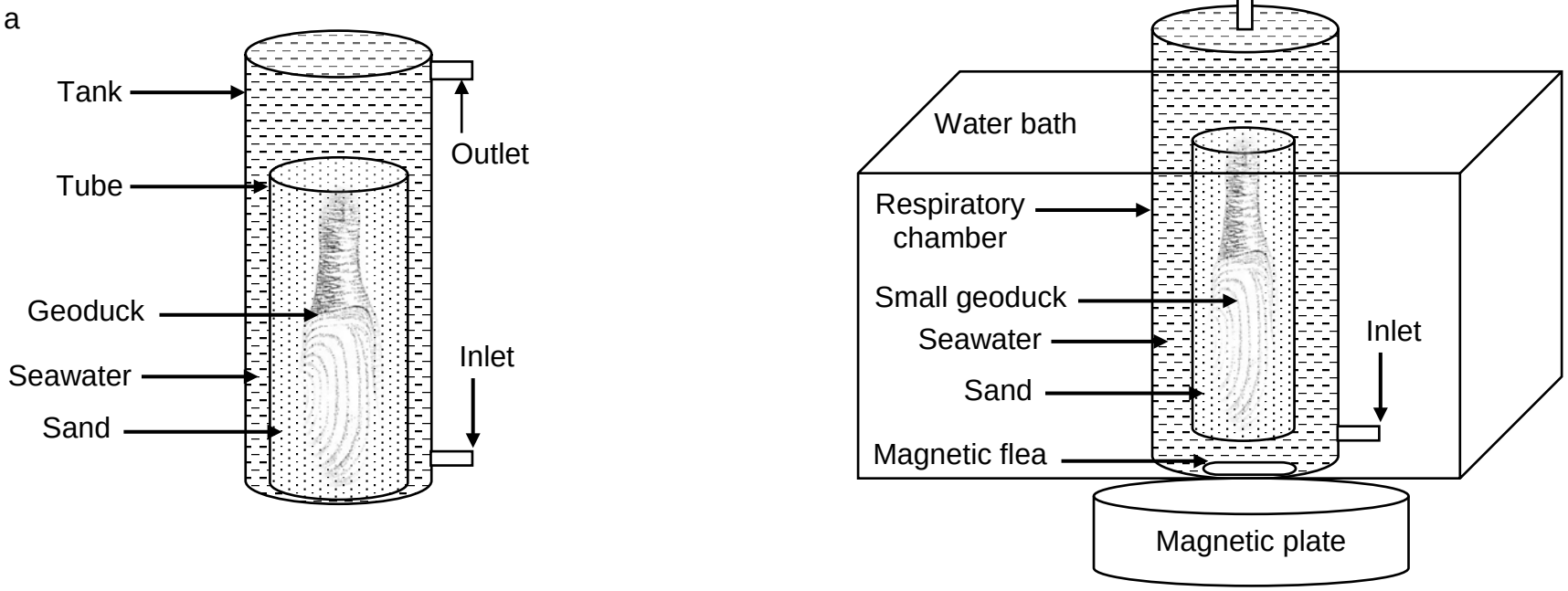
Regression analysis between physiological rates and tissue dry weight

	Clearance rate				Respiration rate			
ANOVA								
Source	DF	Adj SS	F	*P*	DF	Adj SS	F	*P*
logDW	1	5.01	82.23	0.001	1	7.09	731.87	0.001
Error	13	0.79			13	0.13		
Total	14	5.80			14	7.22		
Coefficients								
Term	Coef	SE Coef	T	*P*	Coef	SE Coef	T	*P*
Constant	-0.34	0.08	-4.32	0.001	-0.47	0.03	-15.09	0.001
logDW	0.62	0.07	9.07	0.001	0.73	0.03	27.05	0.001

Regression analysis between physiological rates and shell length

	Clearance rate				Respiration rate			
ANOVA								
Source	DF	Adj SS	F	*P*	DF	Adj SS	F	*P*
logSL	1	4.85	66.01	0.001	1	7.01	438.55	0.001
Error	13	0.95			13	0.21		
Total	14	5.80			14	7.22		
Coefficients								
Term	Coef	SE Coef	T	*P*	Coef	SE Coef	T	*P*
Constant	-3.19	0.41	-7.81	0.001	-3.91	0.19	-20.50	0.001
logSL	1.89	0.23	8.12	0.001	2.27	0.11	20.94	0.001

Figure 7.1 Diagram of a) tank setups used for algal clearance measurements; b-c) respirometer setups used for small (b) and medium/large (c) geoducks



c

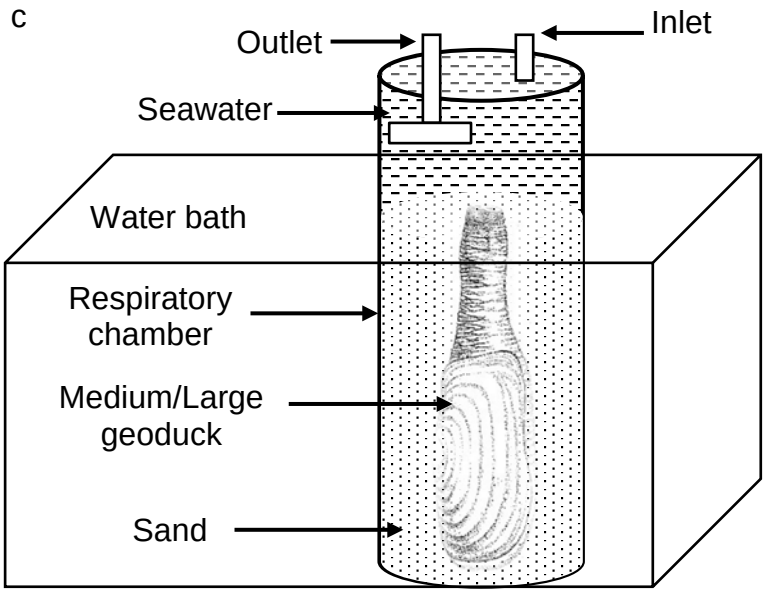
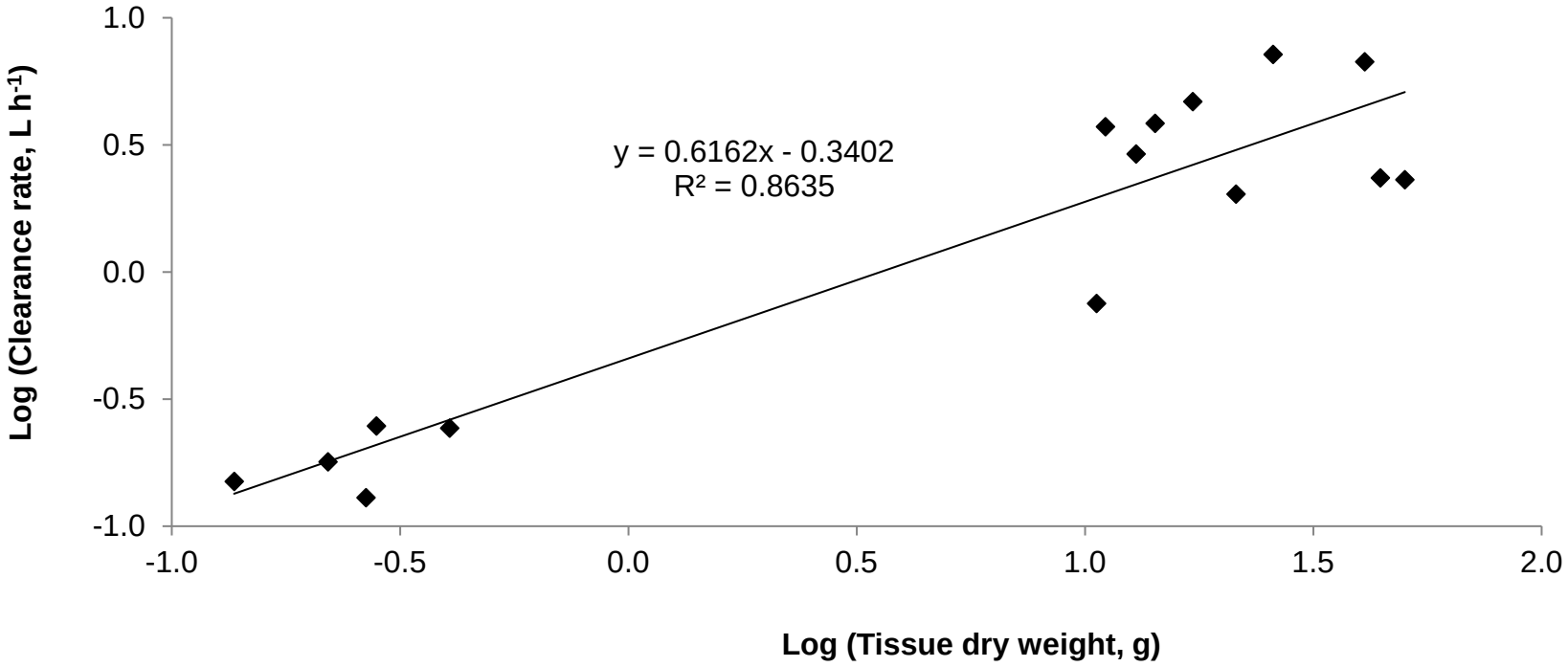
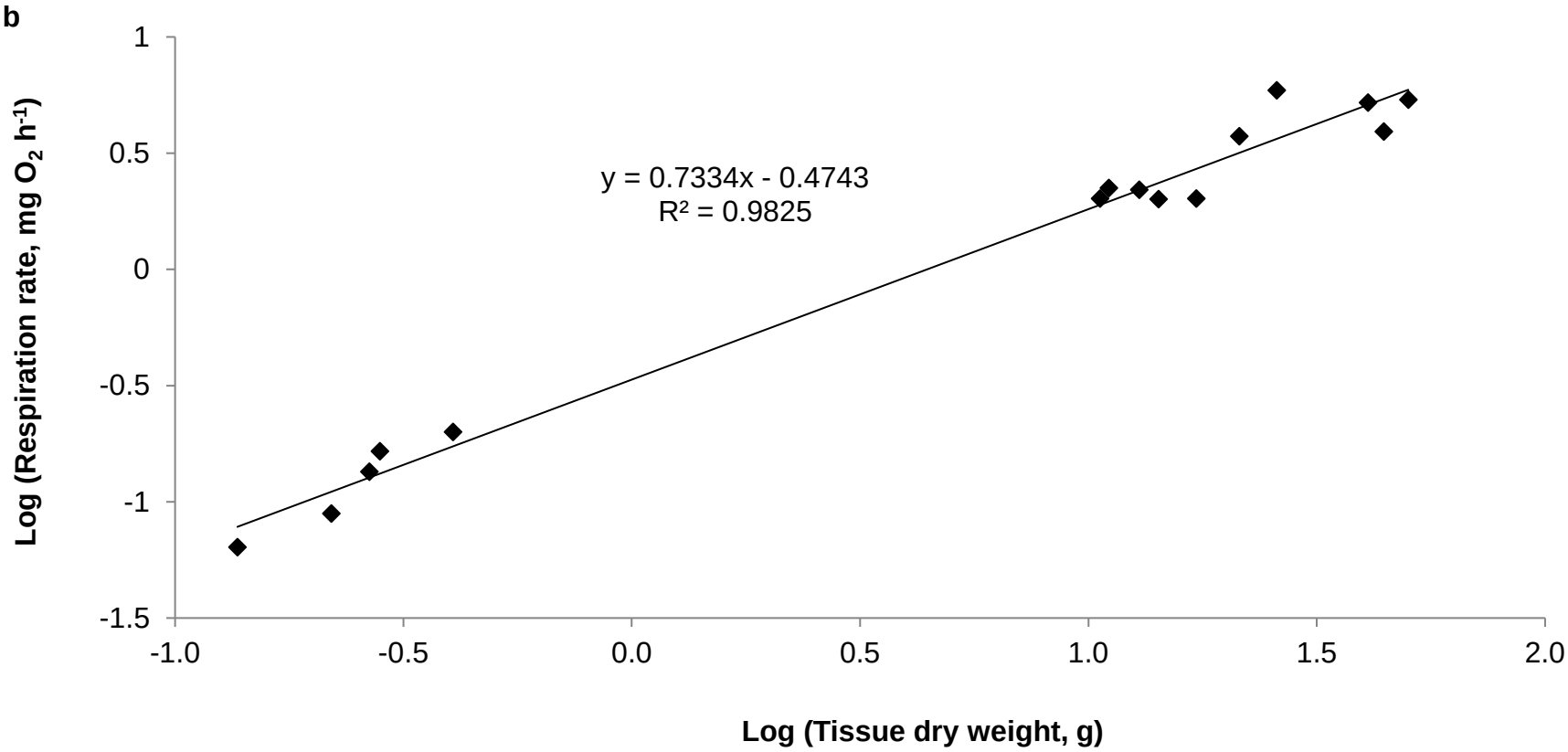

Outlet
Inlet
Seawater
Water bath
Respiratory chamber
Medium/Large geoduck
Sand

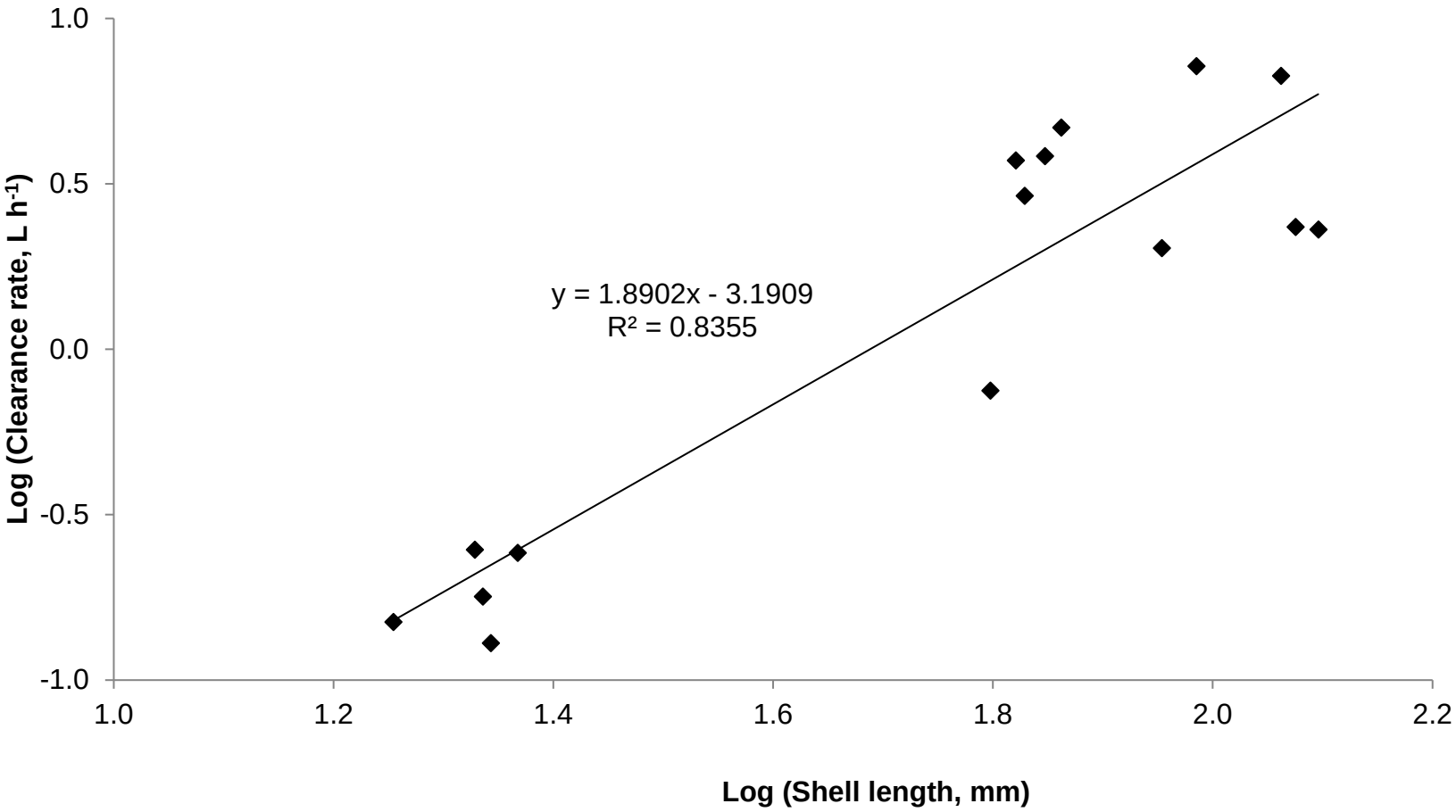
Figure 7.2 Log-log plots describing the relationship between a) clearance rate and b) respiration rate and tissue dry weight; c) clearance rate and d) respiration rate and shell length of *Panopea zelandica*. n = 15.

a

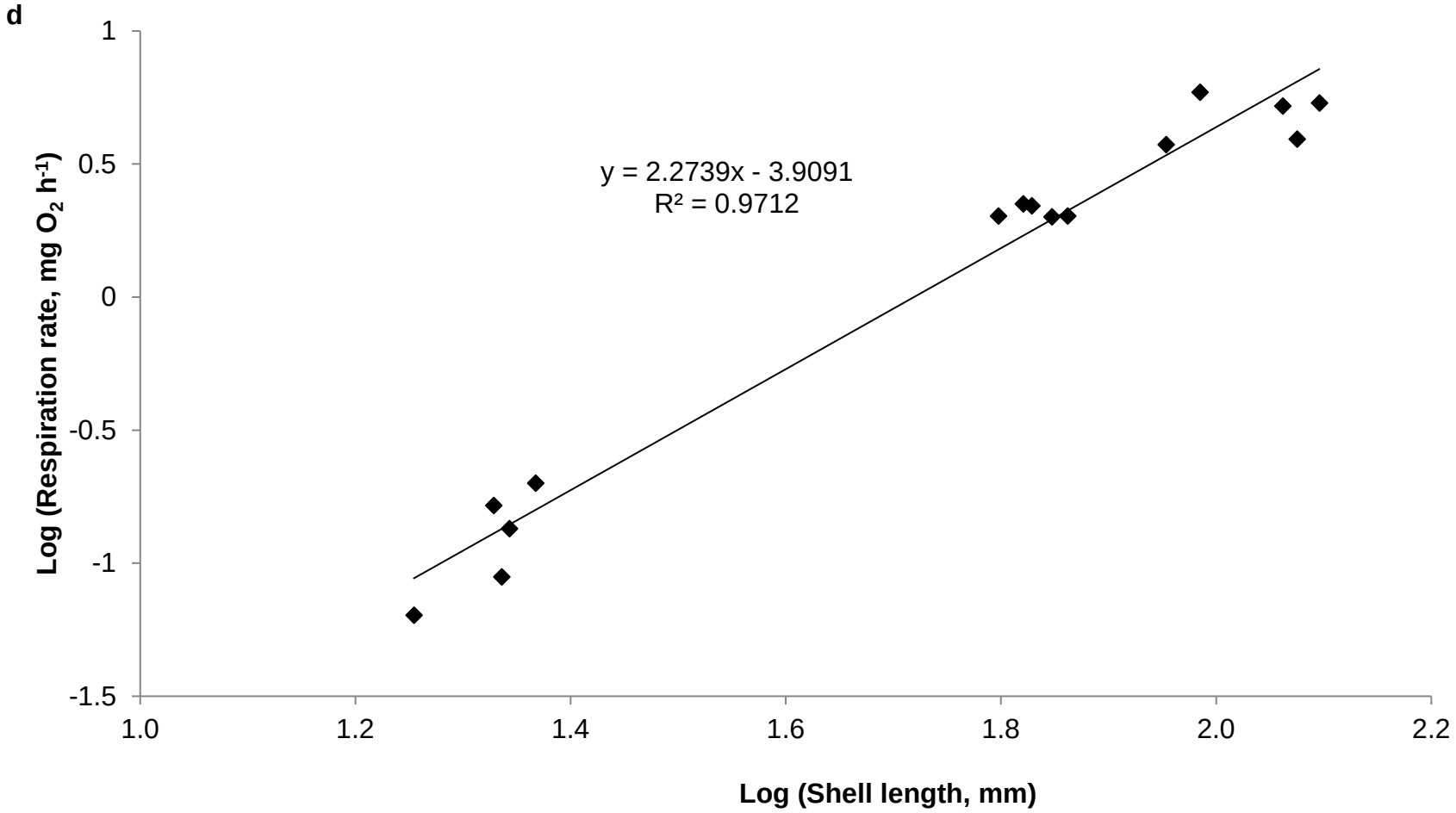



b
Log (Respiration rate, mg O2 h-1)
y = 0.7334x - 0.4743
R² = 0.9825
1
0.5
0
-0.5
-1
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
Log (Tissue dry weight, g)

c


1.0
0.5
0.0
-0.5
-1.0
Log (Clearance rate, L h-1)
y = 1.8902x - 3.1909
R² = 0.8355
1.0
1.2
1.4
1.6
1.8
2.0
2.2
Log (Shell length, mm)

d


y = 2.2739x - 3.9091
R² = 0.9712
Log (Respiration rate, mg O2 h-1)
Log (Shell length, mm)
1
0.5
0
-0.5
-1
-1.5
1.0
1.2
1.4
1.6
1.8
2.0
2.2

CHAPTER 8 - Establishing the thermal window for aerobic scope in New Zealand geoduck clams (*Panopea zelandica*)

This chapter was submitted to Journal of Comparative Physiology B as:

Le, D.V., Alfaro, A.C., Ragg, N.L.C., Hilton, Z., King, N. Establishing the thermal window for aerobic scope in New Zealand geoduck clams (*Panopea zelandica*).

Abstract

Geoduck clams (*Panopea* spp.) are the longest-lived and largest deep burrowing bivalve. Their unique morphology allows them to live buried in the sediment at depths of up to 1m. The endemic New Zealand geoduck (*Panopea zelandica* Quoy and Gaimard, 1835) has recently been identified as a potential species for aquaculture. However, very little is known about the biology and physiology of this entirely sub-tidal geoduck species. Currently, the New Zealand geoduck fishery relies entirely upon wild harvests, but farms are expected to emerge as cultivation protocols are established. A key step in the optimisation of cultivation procedures is the identification of optimal temperature and food rations. One method for establishing thermal optima is to identify the temperature window that supports the widest aerobic scope: the degree to which metabolic rate can be increased to support elevated activity demands. Thus, we investigated the aerobic scope for activity at five different temperatures representative of typical environmental conditions (8, 11, 15, 19, and 23°C) for juvenile and young adult *P. zelandica*. Clearance rate was also measured at all temperatures. Comparisons of aerobic scope for activity and clearance rates between size classes revealed that juvenile geoducks had a narrower thermal optimum than young adults (15 – 19°C *versus* 11 – 19°C, respectively). Temperatures higher than 19°C resulted in a reduction of aerobic scope for activity and clearance rate for both juvenile and young adults, which may lead to reduced performance and elevated mortality. These findings provide the first measures of aerobic scope in *P. zelandica*, a key step towards a meaningful understanding of the ecophysiology of this unusual species.

8.1 Introduction

Geoduck clams (*Panopea* spp.) are the largest burrowing clams worldwide, weighing up to 3.25 kg, and burrowing to depths of 1 m (Goodwin and Pease 1989). Their fused siphons and mantle are too big to retract into their shells, and can extend up to 1 m to reach the sediment surface (Campbell et al. 1998). Adult geoducks have a relatively small foot, and are unable to re-bury themselves if disturbed (Goodwin and Pease 1989). Thus, while depth of interment increases with size, geoducks bury themselves in the sediment for life, which can be up to 168 y without loss of reproductive viability (Goodwin 1976; Orensanz et al. 2004). These unusual morphological characteristics have led to them being considered as 'elephants of bivalve species' (Goodwin and Pease 1989).

The endemic New Zealand geoduck, *Panopea zelandica* Quoy and Gaimard, 1835, is a strictly subtidal species, distributed along the New Zealand coast from at least 36º to 47º latitude, but with a very patchy known distribution (Gribben and Heasman 2015). Already subjected to commercial wild harvest, this species has been identified as a new species with significant potential to contribute to growth of the New Zealand aquaculture industry, and farms are expected to emerge as cultivation protocols are established. However, very little is currently known on this geoduck's biology and physiology. At present, hatchery-raised juveniles (15-20 mm in shell length) are being produced to experimentally re-seed sandy areas under mussel and oyster farms (K. Heasman unpublished data). Once seeded, wild geoducks are estimated to reach a harvestable size (100-110 mm) within 5 to 10 years (Breen et al. 1991; Gribben and Creese 2005). However, there is a lack of information regarding

optimal temperature ranges for this species to assist both hatchery production and selection of farm sites. Temperature ranges for optimal growth have been reported for juvenile *P. generosa* geoducks (3.22 ± 0.05 mm and 0.54 ± 0.01 mm shell length, Arney et al. 2015). However, optimal growth conditions are usually species-specific (Jobling 1981; Pörtner and Peck 2010). Indeed, *P. generosa* can be found in both intertidal and subtidal areas while *P. zelandica* is strictly subtidal (Gribben and Heasman 2015), which makes it likely that these two species may have very different thermal preferences. More generally, temperature is well-known to be a factor determining range expansions and contractions of animals (Sunday et al. 2011, 2012). Hence, a thorough understanding of the thermal tolerance of geoducks is also essential to predict the ecological impacts of future rises in global temperatures.

Temperature has a profound effect on growth of poikilothermic animals. However, as growth is the net outcome of complex interactions between environmental and metabolic factors, such as food availability, temperature, and metabolic efficiency, direct measurements are needed to define the optimal temperature range for growth under given environmental conditions (Sicard et al. 2006). Alternatively, the temperature range for achieving optimal growth can be estimated by measuring scope for growth (e.g. Tamayo et al. 2013) or aerobic scope for activity (e.g. Fry 1947; Pörtner and Peck 2010) over a range of different temperatures.

Aerobic scope for activity is a measure of the amount of aerobic energy metabolism in excess of the basal rate required to sustain life. Activities here are defined as the use of the energy derived from metabolism for locomotion, feeding, growth or reproduction (Fry 1947). Hence, the aerobic scope for

activity is normally calculated as the difference between the highest (active) metabolic rate and the lowest (standard or basal) metabolic rate (Fry 1947; Claireaux and Lefrançois 2007, Pörtner and Peck 2010). In some bivalves, 'active' metabolic rate can be measured during activities such as re-burying (Peck et al. 2004; Lurman et al. 2014a, 2014b) or escape responses (Peck et al. 2004; Schalkhausser et al. 2014). However, in a bivalve with a completely sedentary sessile ecology such as the geoduck where, as adults, even re-burying does not occur and thus no representative major muscular excertion can be elicited from the animal. The only significant 'activity' that is representative of the natural state is the increase in metabolic rate associated with feeding, including the metabolic cost of water processing (Bayne et al. 2001; Norkko et al. 2005; Ibarrola et al. 2008). In this case, an approximation of aerobic scope for activity may be obtained by distinguishing between non-feeding (standard or basal) metabolism, and feeding (active or routine) metabolism (Thompson and Bayne 1972; Lurman et al. 2013). Lurman et al. (2013) found in the sessile mussel *Perna canaliculus* that feeding behaviour elicited a much higher metabolic rate than did the activity of byssus production if animals were dislodged from their substrate. However, the combination of both byssus production and feeding after fasting elicited an even higher rate (Lurman et al. 2013). Thus although the measure of scope for activity derived from feeding activity cannot be proven to be maximally attainable aerobic scope, in the absence of any other major muscular activity, this measure is likely to be the most representative proxy for aerobic scope available to establish the thermal window for activity in geoduck. In this study, we therefore estimated the thermal window for 'aerobic scope' by measuring differences between standard (or

basal) metabolism while fasting, active metabolism during acute feeding immediately after fasting, and routine metabolism during steady-state feeding conditions (Bayne et al. 1976; Lurman et al. 2013).

The thermal window for aerobic scope for activity may be determined according to the animal's acute response to changes in temperature (Bayne et al. 1976; Sokolova and Pörtner 2003; Schulte 2015). Furthermore, the temperature sensitivity of aerobic scope may differ throughout the life stages of an organism (Widdows 1973) or in different-sized individuals (Peck et al. 2007). Although geoducks are known to be extremely long-lived (Orensanz et al. 2004), previous studies have demonstrated that *P. zelandica* may reach reproductive maturity after just 2 years (57.8 ± 5.5 mm shell length; Le et al. 2014). While juvenile geoducks may expend energy on building up somatic tissues, reproductively maturing young adults invest energy in both somatic and reproductive growth. It has been shown that reproduction may have a great effect on aerobic scope for activity (Brokordt et al. 2000). Hence, the optimal temperature ranges for growth of both juvenile and mature young adult *P. zelandica* need to be determined to inform optimal conditions within aquaculture settings, as well as contributing to a better understanding of the effects of temperature changes on recruitment and survival of wild geoduck populations. Thus, the aim of this study is to determine the aerobic scope for activity of juvenile and young adult geoducks to better understand the relationship between ambient temperature and geoduck physiology and distribution.

8.2 Materials and Methods

8.2.1 Animals

All geoducks used in this study were spawned and reared at the Cawthron Institute, Nelson, New Zealand. Juvenile (four-month old) and young adult (three-year old) geoducks were used in two separate experiments. The first experiment included juveniles of 15.89 ± 1.49 mm in shell length and 1.61 ± 0.50 g live weight. The second experiment included young adults of 62.97 ± 4.38 mm in shell length and 118.94 ± 24.32 g live weight. All animals were maintained in a flow-through system with 1 µm filtered seawater at 15°C. Each animal was individually buried in sand in 50 mL Falcon™ tubes (for juveniles) or 700 mL nylon tubes (for young adults) to minimize subsequent handling stress and to provide a stable environment throughout the experiments. Geoducks were positioned at the bottom of the tubes, under approximately 80 mm (juveniles) or 150 mm (adults) of sediment and did not move perceivably once settled. Apart from feeding activity, the only other physical activity observable in geoducks of these ages could be the retraction or extension of their siphons under stress conditions (e.g. predator, hypoxia). However, the animals in our experiments did not appear to retract or extend their siphons at any point.

8.2.2 Experimental design

The experimental temperature range was chosen based on the reported mean sea surface temperatures across the areas of wild geoduck distribution (Gribben et al. 2004) with two additional upper and lower levels, chosen to be relevant for cultivated geoducks in nurseries (Le, pers. obs).

Two different acclimation periods were used for the two size classes; since smaller juveniles are likely to display more rapid physiological acclimation than adults, a shorter acclimation and fasting period was used for juveniles to avoid animals reaching a state of metabolic depression (Pörtner 2014).

Five groups of 7 juvenile and five groups of 7 young adult geoducks were acclimated sequentially in flow-through systems at 15°C for 14 days and 30 days, respectively. After the acclimation period, each group of 7 geoducks was tested sequentially, but in the same manner as shown in Fig. 8.1 for juveniles, and Fig. 8.2 for young adults, due to the limited number of respirometer chambers available. The interval between each series of treatments was kept to a minimum, 3 days for juveniles and 2 days for young adults. Thus all measurements were made within 15 days for juveniles and 10 days for adults, to avoid temporal/seasonal effects, as well as the influence of growth in juveniles and reproductive development in young adults.

Experiment 1: Juvenile geoducks

Standard metabolic rate (SMR): After the 14-day fasting period, juvenile geoducks were placed in separate closed respirometry chambers (Fig. 8.3) to measure oxygen consumption (see below) at 15°C for a period of 4 h (Fig. 8.1). An additional blank respirometer (including a tube with sand, but no geoduck) was used to record the background oxygen levels in the system and thus control for microbial respiration, and/or micro-algal contribution during routine or active metabolism whilst feeding. These oxygen consumption measurements for each group at 15°C prior to the acute thermal change were used to test if there were any significant differences in metabolic rate between the groups (as

the different groups were measured over a period of 15 d). The temperature in the respiration chambers was then changed to the target temperature over a period of 1 h by slowly flushing fresh 1 µm filtered seawater at the target temperature through the chambers. The experimental groups 1-5, were exposed to 8, 11, 15, 19, and 23°C, respectively. Oxygen consumption was then recorded within closed chambers over a period of 15 h for groups 1 and 2 (8 and 11°C), and for 4 h for groups 3 to 5 (15, 19 and 23°C). These differences in recording time were necessary to avoid hypoxia in the chambers where respiration rates were higher due to the higher temperatures. These oxygen consumption measurements were used to calculate standard metabolic rates after acute thermal challenge at the new temperatures.

Active Metabolic Rate (AMR): After SMR had been measured, seawater which contained 30,000 – 40,000 cells mL^{-1} of *Tisochrysis lutea* (formerly known as *Isochrysis affinis galbana* or *T-ISO clone*; Bendif et al. 2013, 2014) was flushed through the chambers so that geoducks could feed (in the dark) inside the respirometers for 4 h under flow-through conditions. This algal ration was selected according to a pilot study of feeding rates over different algal concentrations at 15 and 23°C, which ensured an *ad libitum* supply of food to the animals (Le, unpublished data). Next, the respiration chambers were closed so that oxygen and algal consumption could be measured for 12 h for groups 1 and 2 and for 3 h for groups 3 to 5. These measurements were used to calculate both active metabolic rates and water clearance rates at the new temperatures.

Thus, both standard and active metabolic rates, as well as clearance rates were calculated for juveniles acutely exposed to five different temperature

regimes. After active metabolic rate measurements, the geoducks were removed and the chambers re-sealed to quantify microbial oxygen consumption in the 'dirty' chambers, including sediment, water and any geoduck waste products. This residual oxygen consumption was found to be negligible at 15°C (data not shown). Similarly, the presence of microalgae in blank chambers displayed no measurable oxygen consumption (data not shown) and therefore considered unlikely to influence the animal respiration measurements.

Experiment 2: Young adult geoducks

Routine Metabolic Rate (RMR): Young adult geoducks were fed a ration of 300,000 – 400,000 cells mL^{-1} of *T. lutea* during a 30 day acclimation period at 15°C (Fig. 8.2). This algal ration was determined as sufficient for maximal feeding (*ad libitum*) in a previous broodstock conditioning experiment (Le et al., 2014). After the acclimation period, the animals were placed in separate flow-through respirometer chambers to measure oxygen and microalgal consumption (see below) for a period of 24 h at 15°C. In addition to the 7 animals in each group, an 8^{th} blank chamber with sediment but no geoduck was used to record the background oxygen levels in the system. To avoid hypoxia in the chambers, the water flow rates were adjusted so that the differences in oxygen concentrations between the outflow and inflow were less than 20%. Oxygen consumption was then measured to test for any differences between groups (as groups were measured sequentially over a period of 10 days) in routine metabolic rates at 15°C. The temperature in the respiration chamber was then changed to the target temperature by flushing fresh seawater through the chambers over a period of 1 h while the microalgal concentration was

maintained constant. Target temperatures for groups 1 to 5 were 8, 11, 15, 19, and 23°C, respectively. After the 1 h period to allow initial acclimation to the new temperature, both oxygen and algal consumption were recorded over 24 h under flow-through conditions. These measurements were used to calculate routine metabolic rates and clearance rates at the new temperatures. Thus, routine metabolic rates and clearance rates were calculated for young adults exposed to five different temperatures.

Standard Metabolic Rate (SMR): After routine metabolic rate and clearance rate measurements were completed, each group of geoducks was fasted for 30 days at 15°C (Fig. 8.2). Following this period, baseline metabolic rate measurements were again conducted to check for any differences between the groups before the temperature change. The chamber temperatures were then changed to 8, 11, 15, 19 and 23°C for groups 1 to 5, respectively and left to acclimate to the new temperature for 1 h. After this period, oxygen consumption was measured in the separate flow-through respirometer chambers for 24 h at the new temperature. The purpose of this final temperature change was to expose the five groups to acute temperature changes without feeding to determine the standard metabolic rate of geoducks within each of the five temperature treatments. It was inferred that the bacterial and microalgal oxygen consumptions in the flow-through system were insignificant as there was no difference in the oxygen concentration of the inflow and outflow of the control chambers over the experimental periods.

8.2.3 Physiological measurements

Oxygen consumption measurements

Respirometers (660 mL volume for juveniles and 1270 mL for young adults) were made of acrylic plastic cylinders (60 mm Ø, 5 mm thick) with two Perspex lids (10 mm thick) sealed with screws and wingnuts. Each chamber had a recessed water inlet (on the bottom) and outlet (on top). Individual Falcon™ tubes with juveniles were suspended inside the chambers and a magnetic flea was put beneath this to mix the water in each chamber (Fig. 8.3). The sediment-filled tubes containing young adults were fitted inside the respirometer chambers without a magnetic flea, relying instead upon the flow-through configuration for mixing. The chambers were then submerged in a thermostated water bath maintaining target temperature ±0.1°C. Water movement inside the chambers containing both juveniles and adults was tested initially with dye to verify adequate mixing to avoid oxygen stratification. In a related study (Le et al. 2016) the permeability of the respiromer systems used in the present trials was tested with self-induced hypoxic water, finding no evidence for oxygen diffusion into the system.

For juvenile geoducks, closed system respirometry was carried out following the sampling procedure of Tamayo et al. (2013). Oxygen partial pressure was measured by anaerobically drawing repeated 1 mL samples with a syringe from each chamber outlet. The corresponding 1 mL of air-equilibrated water that replaced the sample was assumed to have an undetectable influence upon the oxygen content of the 660 mL chamber. The water samples were injected into a flow cell containing a polarographic oxygen electrode (MI 730 Micro-Oxygen electrode, Microelectrodes Inc., Bedford, NH) within a water jacket (Cameron

Instruments, Guelph, Canada) maintained the same temperature as the respirometer. The oxygen sensor was connected to a Cameron Instruments OM 200 Oxygen meter. The experiment was ended before the oxygen tension fell below 80% saturation, following the recommendations of Morley et al. (2007) and Watson et al. (2014). The residual water volumes of the chambers were measured at the end of the experiment. Preliminary trials, using an oxygen sensor mounted within a respirometer, continually recording oxygen tension, indicated the reduction was strongly linear over 15 h (R^2 = 0.98, data not shown). Hence, the oxygen consumption rate of geoducks within normoxia was considered to be constant over time. Determining oxygen consumption over an extended integration period was also considered desirable to avoid signal noise associated with potential variation in ventilation rate (cf. valve closure in other bivalves) or the possibility that the geoduck may periodically utilize anaerobic metabolism, even under normoxia, as observed in other bivalves (e.g. cockles, Wang and Widdows, 1993). Initial and final oxygen tension values were therefore used to establish a mean consumption rate for the entire monitoring period.

In the case of young adults, oxygen measurements were conducted in a flow-through respirometry system (Fig. 8.3). The flow rate was measured and adjusted three times daily to achieve a difference of 10 – 20% in the oxygen concentration between the inflow and the outflow. This was necessary to avoid hypoxia and ensure the reliability of measurements. Short lengths of low permeability tubing (polyurethane high pressure pneumatic, 6mmØOD, 4mmØID, TU0604 LDS.3, SMC Corp. Noblesville, IN) connected the recessed outlets of each chamber to an auto-switch control system. A programmable

logic controller (PLC, Schneider Electric Premium series) was designed to control nine solenoid valves that allowed outflow water from only one chamber to flow through the oxygen meter at any given time. While oxygen measurements were recorded on one chamber, the water in the other chambers flowed to waste. An optical oxygen sensor (FOXY-R, Ocean Optics, Dunedin, FL) within a water-jacketed flow cell was connected to a phase measurement system (Ocean Optics, NeoFox®). This measurement system was connected to the PLC, which was programmed to record the oxygen tension values at hourly intervals for each chamber in turn.

Algal consumption measurements

For juvenile geoducks, the Coughlan method (Coughlan 1969) was used to determine individual algal consumption rates within the closed system. The algal concentrations in the chambers were measured with a Coulter Counter (Beckman Coulter Multisizer 4, gated to 2.72 - 20 µm diameter) at the beginning and at the end of the monitoring period. At the end of the measurement period the final amount of microalgae remaining in the chambers was always over 50% of initial concentration, which has been suggested to be appropriate to assure representative clearance rate estimates (Riisgård 2001). The water volume in the chambers was measured at the end of the experiment.

For young adults, a flow-through method was applied to determine individual algal consumption rates (Bayne et al. 1976). The flow rates in each chamber were monitored and adjusted three times daily to ensure no more than 15 – 25% of the microalgae were cleared by the geoducks. This consumption rate was used to ensure reliability of measurements, whilst ensuring flow rates

were sufficient to prevent water re-filtration by the animals within the chamber (Crisp 1971). Water samples were continuously taken for 24 h from a drip line in the outflow of each chamber and the control chamber. The microalgal concentration was measured using a Coulter Counter as described above.

8.2.4 Calculation of physiological rates

Respiration rate

For juveniles in closed respirometers, oxygen consumption rates were determined following the equation (after Sobral and Widdows 1997):

$$RR = \frac{O_{t0} - O_{t1}}{t} \times V$$

where, RR is the respiration rate (mg O_2 h^{-1}), O_{t0} and O_{t1} are the initial and final oxygen concentrations (mg O_2 L^{-1}) in the chamber, respectively; V is the volume of water (L), and t is the interval between samples (h).

For young adults in a flow-through system, the respiration rates were calculated using the equation (after Moullac et al. 2007):

$$RR = (O_{in} - O_{out}) \times FR$$

where, RR is the respiration rate (mg O_2 h^{-1}), O_{in} and O_{out} are the oxygen concentrations (mg O_2 L^{-1}) of the inflow and outflow, respectively, and FR is the flow rate (L h^{-1}).

Clearance rate

For juveniles in a closed chamber the clearance rates were determined following the equation of Coughlan (1969):

$$CR = \frac{\ln(C_{t0}/C_{t1})}{t} \times V$$

where, CR is the clearance rate (L h^{-1}), C_{t0} and C_{t1} are concentrations of microalgae (cells mL^{-1}) at the start and end of the measuring period, V is the volume of water (L), and t is the sample interval (h).

For young adults on flow-through, the clearance rates were determined following the equation proposed by Filgueira et al. (2006):

$$CR = \frac{C_{in} - C_{out}}{C_{in}} \times FR$$

where, CR is the clearance rate (L h^{-1}), C_{in} and C_{out} are the algal concentrations (cells mL^{-1}) at the inflow and outflow, respectively, and FR is the flow rate (L h^{-1}).

Size standardization

At the end of the experiment, geoduck tissues were removed from their shells and dried at 104°C for 24 h. The physiological rates were then standardized to 1 g of soft-tissue dry weight for both juvenile and young adult geoducks using the formula given by Bayne and Newell (1983):

$$Y_s = Y_e \times (W_s/W_e)^b$$

where, Y_s is the physiological rate of the standard-sized animal, Y_e is the experimentally recorded physiological rate, W_s is the standard tissue dry weight (1 g in this study), W_e is the tissue dry weight of the experimental geoduck (g), and b is the weight exponent: b = 0.62 for clearance rate, and b = 0.73 for respiration rate. Allometric exponents were based on data calculated from

measurements of respiration and clearance rates of *P. zelandica* of different sizes in related experiments (Le et al. in prep).

Aerobic scope for activity

Aerobic scope for activity (SFA) was calculated following the equations given by Bayne et al. (1976):

$SFA = AMR - SMR$ (for juveniles) or $SFA = RMR - SMR$ (for adults)

and factorial aerobic scope as

$FAS = AMR/SMR$ (for juveniles) or $FAS = RMR/SMR$ (for adults)

where, SFA is the aerobic scope for activity, FAS is the factorial aerobic scope, AMR is the standardized active metabolic rate (measured during feeding after fasting), RMR is the standardized routine metabolic rate (measured during routine feeding), SMR is the standardized standard metabolic rate (measured during fasting), and all rates have the unit of mg O_2 g DW^{-1} h^{-1}, except for FAS which is dimensionless.

8.2.5 Statistical analysis

Significant differences in standard, active, and routine metabolic rates, aerobic scope for activity, factorial aerobic scope, and clearance rate associated with temperature exposures were tested with one-way analysis of variance (ANOVA) at a significance level of $\alpha = 0.05$. Statistical analyses were performed using PRIMER v.6. Prior to ANOVA, normality and homogeneity of variances were tested by means of Kolmogorov-Smirnov and Levene's tests respectively.

8.3 Results

There were no significant differences between the 5 experimental groups of geoduck whose physiological parameters were measured at 15°C at different times over the course of the experimental period of 15 days. This was to ensure that animals were not in a state of change (still acclimating, or declining into metabolic depression). And this was not observed. For juveniles, the repeated measures of standard metabolic rate (SMR; mean ± SD) measures were 0.1 ± 0.04 (n=35) for all groups initially and 0.1 ± 0.03 (n=7) for the group tested at 15°C with no significant difference between groups (P = 0.899). For young adults, the measurements for SMR (mean ± SD) were 0.12 ± 0.07 (n=35) for all groups initially, and 0.16 ± 0.05 (n=7) for the group tested at 15°C (P = 0.375). For routine metabolic rate (RMR) they were 0.43 ± 0.1 (n=35) for all groups initially and 0.45 ± 0.1 (n=7) for the group tested at 15°C with no significant difference between groups (P = 0.716).

8.3.1 Experiment 1: Juvenile geoducks

In the juvenile geoducks, the SMR (measured after fasting for 15 d) showed very little temperature sensitivity at temperatures below 15°C, with no significant differences between rates at 8, 11 and 15°C (P = 0.98-0.99) (Fig. 8.4a). However, there was a significant increase above 15°C to 0.23 ± 0.08 mg O_2 g DW^{-1} h^{-1} at 19°C, but interestingly this was not significantly different from the rate of 0.26 ± 0.06 mg O_2 g DW^{-1} h^{-1} measured at 23°C, the highest temperature measured (Fig. 8.4a).

Active metabolic rate (AMR) showed both an identical trend and identical values to SMR at the two lowest temperatures of 8 and 11°C (Fig. 8.4a), and

identical values to SMR at the highest temperature of 23°C (0.26 ± 0.08 mg O_2 g DW^{-1} h^{-1}).

However, AMR showed overall a very different pattern to SMR caused by the mid-range temperatures, with the highest overall values for AMR recorded at 15 and 19°C (0.33 ± 0.15 and 0.34 ± 0.19 mg O_2 g DW^{-1} h^{-1}, respectively).

Thus, absolute values for SFA values were almost zero at the two lowest temperature treatments and the highest temperature treatment, with a peak of SFA occurring at 15°C (0.23 ± 0.13 mg O_2 g DW^{-1} h^{-1}; Fig. 8.4b). A very similar pattern was observed with FAS, where values were around 1 at 8, 11, and 23°C, and FAS peaked at 3.22 ± 1.03 at 15°C (Fig. 8.4c).

Clearance rate (CR) values were similar to patterns of SFA and FAS, with low rates at the lowest two temperatures, with a maximum peak at 15°C of 0.56 ± 0.29 L g DW^{-1} h^{-1}, and declining thereafter within the highest temperatures, but remaining higher (but not significantly so) than the rates at the two coldest temperatures (Fig. 8.4d).

8.3.2 Experiment 2: Young adult geoducks

For young adult geoducks, the SMR (measured after fasting for 15 d) was extremely low, at 0.02 ± 0.01 mg O_2 g DW^{-1} h^{-1} at 8°C, but increased virtually linearly with increasing temperature to a maximum of 0.29 ± 0.12 mg O_2 g DW^{-1} h^{-1} at the highest temperature of 23°C (Fig. 8.5a).

The pattern for RMR was that of increasing values from 0.18 ± 0.003 mg O_2 g DW^{-1} h^{-1} at 8°C to a peak of 0.26 ± 0.07 mg O_2 g DW^{-1} h^{-1} at 19°C, followed by a decrease at 23°C to values similar to those at 15°C (Fig. 8.5a).

The resulting SFA values thus followed an increasing trend with increasing temperature towards a maximum value of 0.36 ± 0.16 mg O_2 g DW^{-1} h^{-1} at 19°C, followed by a sharp decline at 23°C to values not significantly different from those at 8°C (Fig. 8.5b).

Conversely FAS was extremely high at 8°C (15.40 ± 11.01) owing to the very small denominator, and then was significantly lower at all higher temperatures (*P* = 0.007 - < 0.001; Fig. 8.5c).

CR values also followed a similar trend to that of SFA with a peak of 0.74 ± 0.18 L h^{-1} g DW^{-1} at 19°C, which was not significantly different to that at 15°C, and a lowest value of 0.29 ± 0.15 at 23°C which was not significantly different to that at 11°C (Fig. 8.5d).

8.4 Discussion

This study presents the first thermal performance curves for the New Zealand geoduck *P. zelandica*, based on aerobic scope for activity, and uses CR as another measure to estimate the thermal window for activity in this species, as a first step towards establishing thermal optima and critical limits for this bivalve.

Juvenile geoduck had a narrow thermal window of scope for activity (SFA) spanning at most 12°C, as there was essentially zero SFA at temperatures of 11 and 23°C, whilst young adults had a somewhat broader thermal window, and maintained at least some SFA across all temperatures measured (between 8 and 23°C). Juveniles showed a peak of SFA at 15°C (the acclimation temperature), whilst young adults peaked in SFA at 19°C and exactly the same pattern of peak performance was seen in both size/age

classes when clearance rate was examined. The range of temperatures examined in this study (8 - 23°C) correspond to the minimum and maximum temperatures that *P. zelandica* are likely to encounter in their known distribution range along the coast of New Zealand from at least 36º to 47º of latitude (Gribben and Heasman 2015). However, the narrower thermal window demonstrated in this study, is in accordance with the narrower range of temperatures of 11 – 19°C recorded in bays where wild *P. zelandica* adults were actually found (Gribben et al. 2004). *P. zelandica* adults have been reported to spawn when seawater temperatures are 14 – 16°C in the wild, and juveniles are likely to grow at temperatures of 15 - 19°C in the following months (Gribben et al. 2004).

Although our experiments do not provide biochemical information, juvenile geoducks used in other related experiments were observed to have higher growth rates (as measured by shell length and live mass gain) when reared at 15°C compared to lower temperatures (7 – 11°C), and high mortality was observed at 23°C (Le et al. unpublished data).

In addition, the SFA values near zero at 8, 11, and 23°C, suggest that ~11°C is likely to be a critical lower temperature boundary for growth, while 23°C represents, or perhaps slightly exceeds the upper limit of the thermal range beyond which aerobic capacity, and presumably growth, may not take place for juvenile geoducks.

Clearance rates in our study showed the same thermal response pattern as did absolute aerobic scope for activity. It did not correlate however, with factorial aerobic scope. The decline in the feeding activity in bivalves exposed to temperature beyond their optimum has been observed in other bivalves such

as juvenile *Ostrea edulis* oysters (Buxton et al. 1981) and juvenile *Venerupis corrugata* clams (Albentosa et al. 1994). It is suggested that this decrease in the clearance rate is a direct result of the decline in the SFA. Hence, when the aerobic scope is limited, the feeding activity is compromised. Higher scope for growth or weight gain has been directly correlated with increasing food ingestion rate for *Venerupis pullastra* (Albentosa et al. 1994), juvenile *Mytilus edulis* (Winter 1978), and *O. edulis* spat (Beiras et al. 1994). It is therefore likely that reduced ingestion, as well as increased SMR, will cause a decrease in growth rates at elevated temperatures. A significant decrease in aerobic scope for activity when exposed to acute thermal change to 23°C in this study, and high mortalities observed in young adult geoducks chronically exposed to 23°C in a related study (Le et al. unpublished data), suggest that the optimal temperature for SFA of 19°C may be quite close to the critical and lethal temperatures for this species when exposed chronically.

While species-specific optima vary, the correlation between feeding activity and SFA or scope for growth is also clear in other studies of bivalves (*Ruditapes philippinarum* and *Musculista senhousia* adults, Sgro et al. 2005; adult *Crassostrea corteziensis*, Guzmán-Agüero et al. 2013). Likewise, similar aerobic scope for activity bell-curves have been reported for other adult bivalves such as mussel *M. edulis* (Widdows 1973; Bayne et al. 1976), *Crassostrea gigas* (Le-Gall and Raillard 1988) and the clam *Ruditapes philippinarum* (Han et al. 2008).

The reduction in CR at high and low temperatures recorded for young adult geoducks could be explained by a combination of mechanical and physiological effects. For example, decreasing water temperature results in

higher viscosity, which increases the resistance of water flow created by the cilia, and consequently may reduce the clearance rate of ciliary filter-feeders, such as bivalves (Kittner and Riisgård 2005; Larsen and Riisgård 2009). In addition, geoducks react to adverse conditions, including low or high temperature, by partial or complete closure of their siphon holes, which in turn prevents or reduces water pumping. Reduction in clearance rates has been shown to be a periodic feeding behaviour in response to warming conditions in other bivalve species, such as the mussel *M. galloprovincialis* (Anestis et al. 2010) and the clams *Mercenaria mercenaria* and *R. philippinarum* (Laing et al. 1987).

The aerobic scope for activity results from this study suggest a wider optimal temperature range for young adult (11 – 19°C) than for juvenile geoducks (15 - 19°C). This pattern fits well with the hypothesis that the width in thermal window changes across different life stages for a given species (Pörtner and Farrell 2008), and that for marine invertebrates, juveniles tend to have lower thermal tolerances than their conspecific young adults (Kinne 1970). Also, our results on thermal tolerance and our published oxygen regulation capacity data (Le et al. 2016) for juvenile and young adult geoducks support the concept that poor respiratory control dictates a narrow optimal temperature range (Verberk and Bilton 2013).

Within an aquaculture context, the results of this study provided baseline information for farming geoducks more effectively. Since juvenile geoducks have a narrower optimal thermal range, it is suggested that they should not be planted during winter (<15°C) in the South Island or during summer (>19°C) in the North Island in New Zealand. The wider optimal thermal ranges of young

adults means that they could grow well in coastal areas of New Zealand where local water temperatures do not exceed 19°C or drop below 11°C.

Some compromise is unavoidable in any practical examination of metabolic scope. In the current study acute temperature changes were applied to allow metabolic rate differences to be established for an individual, without the compounding factors associated with extending the interval between measurements. It would therefore be useful to establish the degree to which extended acclimation influences metabolic responses to temperature. In the absence of baseline information, the present study was conducted over a coarse thermal gradient; subsequent fine-scale investigation of responses in the thermal bands approaching critical tolerance limits would be of significant ecological relevance. A logical and reasonably repeatable method to induce maximal activity in burrowing bivalves is disinterment, inducing re-burying behaviour (e.g. Lurman et al. 2014a). However *P. zelandica* show no inclination to re-burrow, nor do they exhibit other repeatable muscular exertion. To establish a representative, repeatable approximation of active metabolic rate, the present study therefore relies upon feeding activity. It is likely that subtle muscular activity occurring during feeding could further elevate metabolic rate, as observed in the mussel, *Perna canaliculus*, where foot movement increased AMR beyond that of feeding alone by approximately 8% (Lurman et al. 2013). The values reported here may therefore slightly underestimate true aerobic scope.

In summary, this study presents the thermal windows for aerobic scope of both *P. zelandica* juveniles and young adults when exposed to acute thermal challenge at a range of temperatures relevant to the extremes of temperature

likely to be experienced in New Zealand coastal waters. While these thermal windows differed between size classes, the patterns for scope for activity and clearance rate were closely correlated, and thus, our study is an important first step in identifying the optimal temperature ranges for growth of juvenile and young adult *P. zelandica* (at between 15 - 19°C and 11 – 19°C, respectively). Temperatures higher than 19°C resulted in a large reduction of aerobic scope for activity for both juvenile and young adults, which may lead to mortality if experienced over extended periods. Hence, for aquaculture, grow-out site selection and out-planting practices should therefore aim to accommodate the narrower thermal window of the juvenile stage. With significant increases in mean and maximum sea surface temperature projected for the coming decades (IPCC 2014) the availability of thermally suitable habitat for geoduck is likely to decline unless suitable adaptation occurs. Further investigation of the phenotypic plasticity and evolutionary potential of this enigmatic and potentially vulnerable species would therefore be of value.

8.5 References

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Figure 8.1 Experimental design to establish the thermal window of aerobic scope and clearance rate for juvenile *P. zelandica*. SMR = standard metabolic rate, AMR = active metabolic rate.

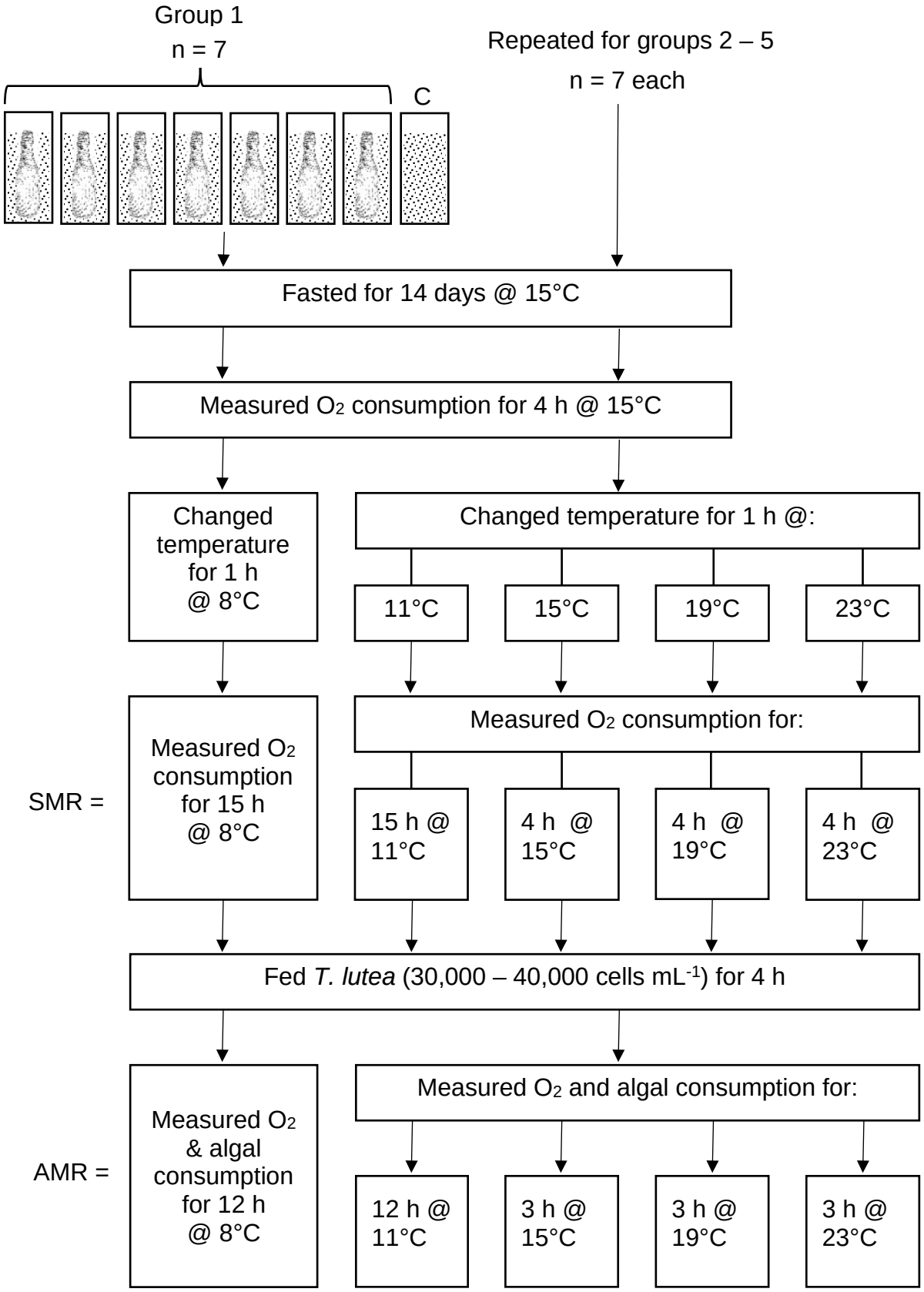

Group 1
n = 7
C
Repeated for groups 2 – 5
n = 7 each
Fasted for 14 days @ 15°C
Measured O2 consumption for 4 h @ 15°C
Changed temperature for 1 h @ 8°C
Changed temperature for 1 h @:
11°C
15°C
19°C
23°C
SMR =
Measured O2 consumption for 15 h @ 8°C
Measured O2 consumption for:
15 h @ 11°C
4 h @ 15°C
4 h @ 19°C
4 h @ 23°C
Fed T. lutea (30,000 – 40,000 cells mL-1) for 4 h
AMR =
Measured O2 & algal consumption for 12 h @ 8°C
Measured O2 and algal consumption for:
12 h @ 11°C
3 h @ 15°C
3 h @ 19°C
3 h @ 23°C

Figure 8.2 Experimental design to establish the thermal window of aerobic scope and clearance rate for young adult *P. zelandica*. RMR = routine metabolic rate, SMR = standard metabolic rate.

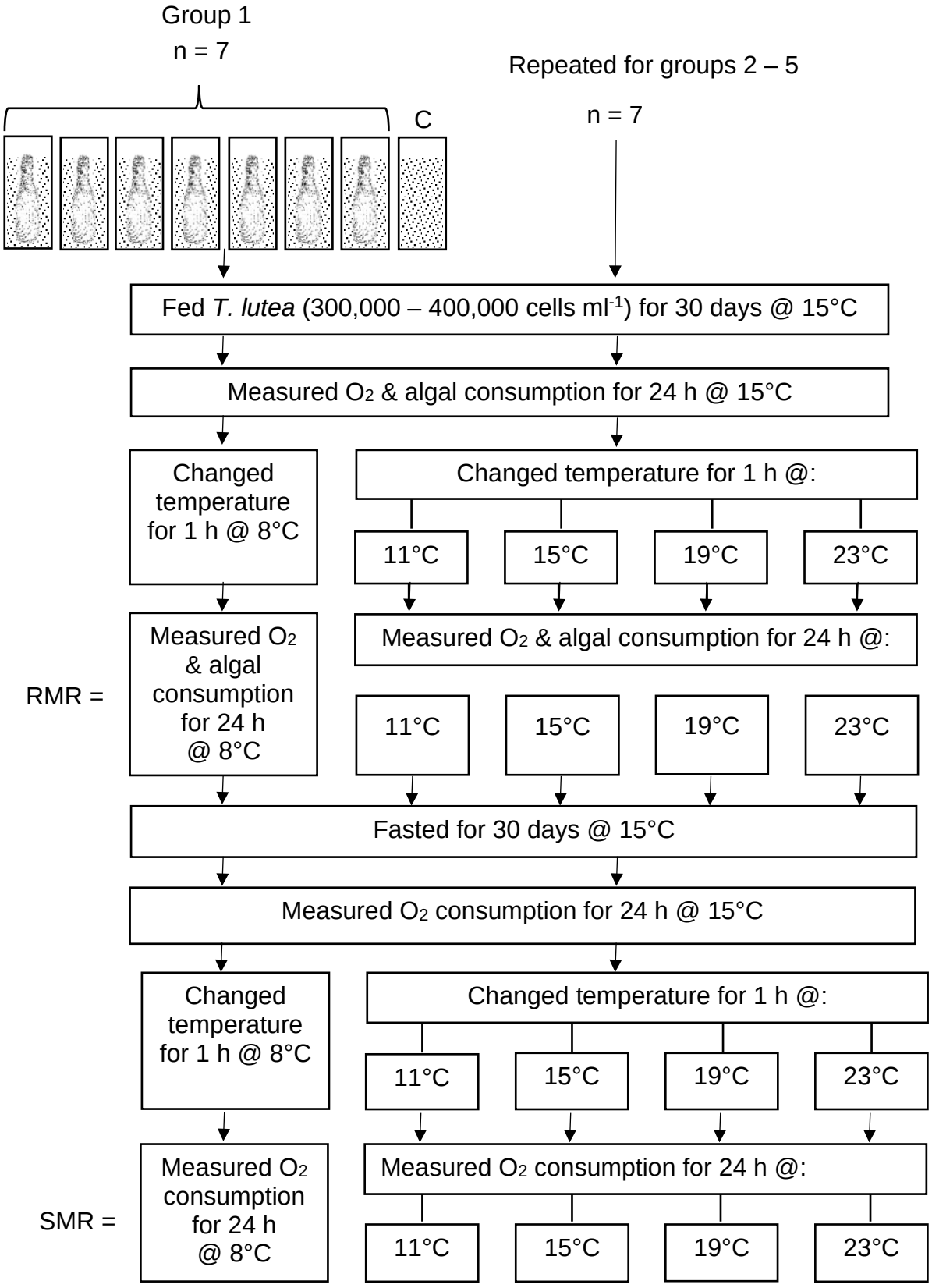

Group 1
n = 7
C
Repeated for groups 2 – 5
n = 7
Fed T. lutea (300,000 – 400,000 cells ml-1) for 30 days @ 15°C
Measured O2 & algal consumption for 24 h @ 15°C
Changed temperature for 1 h @ 8°C
Changed temperature for 1 h @:
11°C
15°C
19°C
23°C
RMR =
Measured O2 & algal consumption for 24 h @ 8°C
Measured O2 & algal consumption for 24 h @:
11°C
15°C
19°C
23°C
Fasted for 30 days @ 15°C
Measured O2 consumption for 24 h @ 15°C
Changed temperature for 1 h @ 8°C
Changed temperature for 1 h @:
11°C
15°C
19°C
23°C
SMR =
Measured O2 consumption for 24 h @ 8°C
Measured O2 consumption for 24 h @:
11°C
15°C
19°C
23°C

Figure 8.3 Diagram of respirometer setups used in the juvenile (left) and young adult (right) experiments.

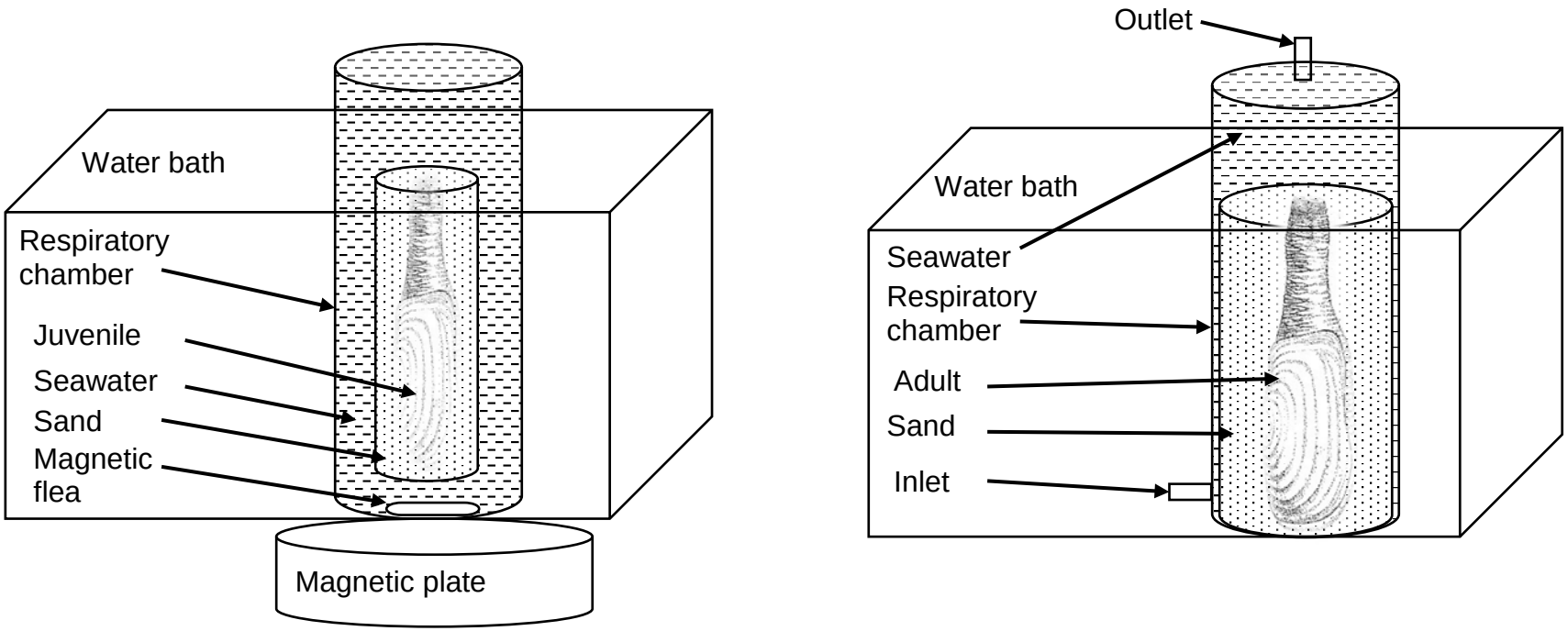


Figure 8.4 a) The standard and active metabolic rates, b) aerobic scope for activity, c) factorial aerobic scope and d) clearance rates of juvenile *P. zelandica* at different temperatures. Data plotted as mean (± SD), n = 7. Distinct letters along the lines indicate significant differences in mean values between temperatures ($p < 0.05$).

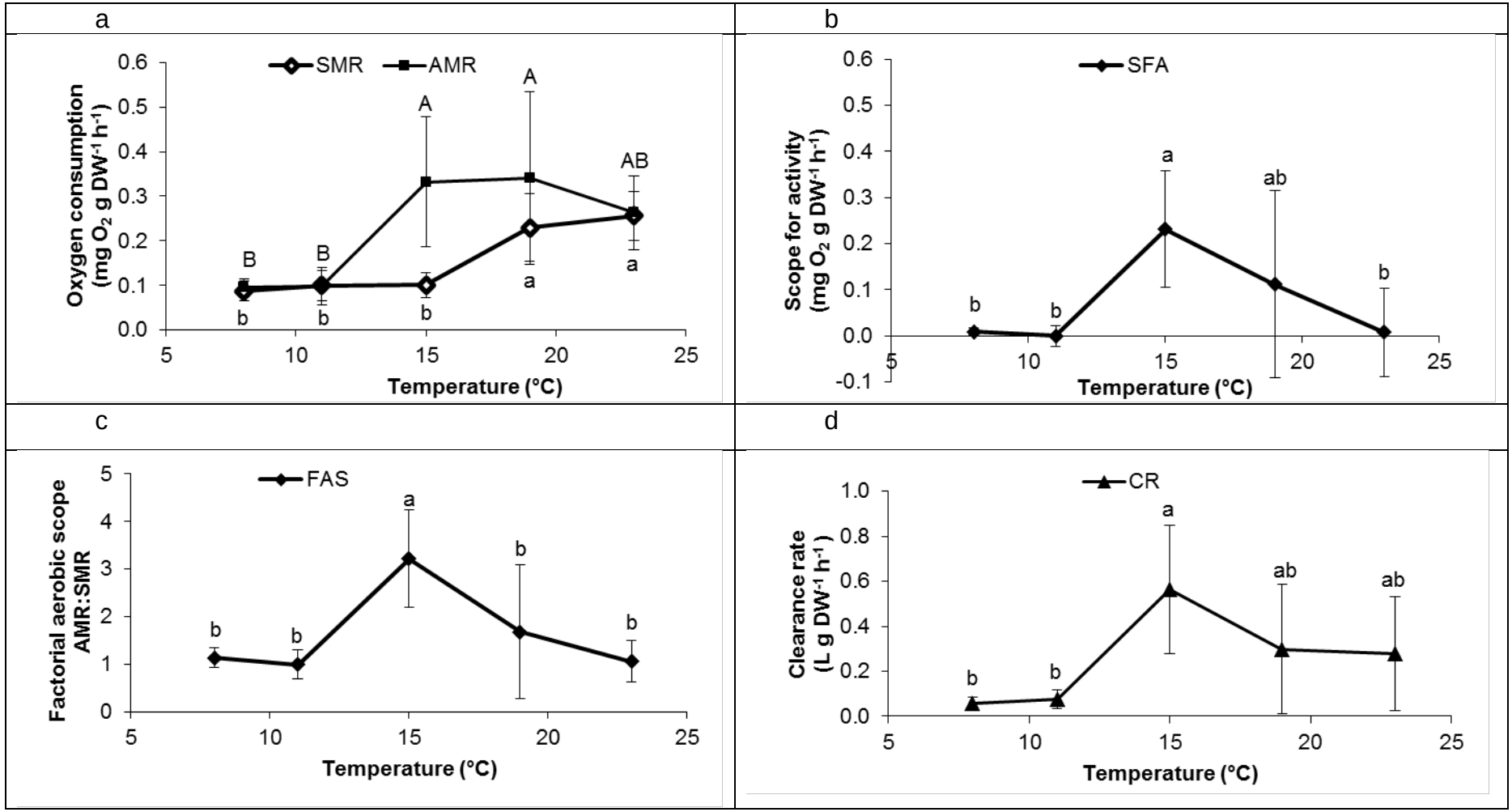
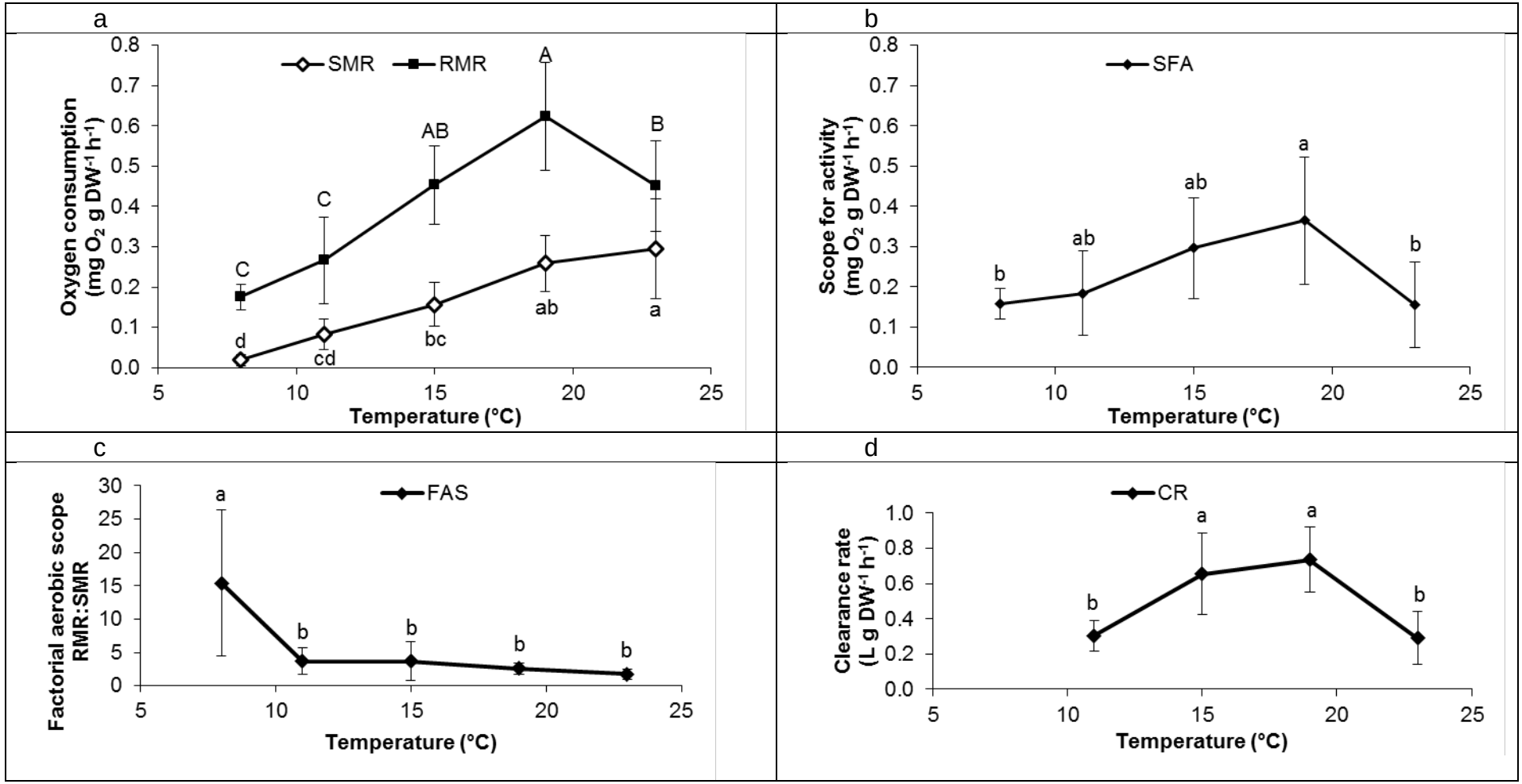

a
SMR
AMR
Oxygen consumption (mg O_2 g DW^{-1} h^{-1})
Temperature (°C)
b
SFA
Scope for activity (mg O_2 g DW^{-1} h^{-1})
Temperature (°C)
c
FAS
Factorial aerobic scope AMR:SMR
Temperature (°C)
d
CR
Clearance rate (L g DW^{-1} h^{-1})
Temperature (°C)

Figure 8.5 a) The standard and routine metabolic rates, b) aerobic scope for activity, c) factorial aerobic scope and d) clearance rates of young adult *P. zelandica* at different temperatures. Data plotted as mean (± SD), n = 7. Distinct letters along the lines indicate significant differences in mean values between temperatures ($p < 0.05$).


a
SMR
RMR
Oxygen consumption (mg O_2 g DW^{-1} h^{-1})
Temperature (°C)
b
SFA
Scope for activity (mg O_2 g DW^{-1} h^{-1})
Temperature (°C)
c
FAS
Factorial aerobic scope RMR:SMR
Temperature (°C)
d
CR
Clearance rate (L g DW^{-1} h^{-1})
Temperature (°C)

CHAPTER 9 – Aerobic scope and oxygen regulation of New Zealand geoduck (*Panopea zelandica*) in response to progressive hypoxia

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Abstract

Recent efforts to cultivate subtidal New Zealand geoducks (*Panopea zelandica* Quoy & Gaimard, 1835) in intertidal areas have been challenged due to daily hypoxia. Hence, further understanding of physiological responses of geoducks of different sizes under different oxygen partial pressures (PO_2) is required to optimize their husbandry. Without this knowledge, the risk of increasing cultivation costs due to growth depression and mortality may increase in both magnitude and frequency. Hence, this study investigated the respiration, aerobic scope, critical oxygen partial pressure (P_cO_2), and oxygen regulation capacity from normoxia to hypoxia in two size classes of fed and starved *P. zelandica*. The P_cO_2 was determined to be ~4 kPa for all geoduck groups. The respiration rates of fed small geoducks decreased significantly from normoxia (16.7 - ~21 kPa) to mild hypoxia (P_cO_2 - 16.7 kPa). Conversely, the respiration rates of starved geoducks from both size classes, and large fed geoducks were maintained at a constant level when exposed to the same change in oxygen concentration. However, all geoducks experienced decreased respiration rates during severe hypoxia (0 kPa - P_cO_2). In addition, overall oxyregulatory capacity, assessed using a regulation index, was affected by size rather than by nutritional stress. Large geoducks maintain oxygen consumption across an oxygen gradient more effectively than small geoducks. Also, the aerobic scope of small geoducks decreased significantly with declining PO_2, while large geoducks maintained their aerobic scope under hypoxia. Hence, to avoid stress and maximize growth potential of cultivated geoducks, smaller individuals should be grown in areas that remain normoxic, whereas larger individuals may

be transferred to on-growing sites that experience periodic hypoxia, potentially including tidal emersion.

9.1 Introduction

Hypoxia can have a major effect on the growth of some aquatic organisms and is responsible for significant losses in aquaculture and fisheries (Díaz et al., 2012). Massive mortality of shrimp and fish in closed intensive culture systems (tanks/ponds) is known to result from oxygen depletion events, which often take place in early mornings when planktonic algae consume oxygen at a high rate (Erez et al., 1990; Szyper et al., 1992). This imbalance between oxygen consumption and production also has been observed in summer and autumn in open water areas where fish cages are located (Yoshikawa et al., 2007). Hence, oxygen is generally carefully monitored and controlled in intensive aquaculture systems (Boyd and Tucker, 1998). However, oxygen concentrations may not be easily regulated in coastal areas, and in some areas eutrophication is a major environmental problem. In such areas, collapses in bivalve aquaculture production and fisheries have been reported (Díaz et al., 2012). For example, hypoxia has been implicated as the key agent eliminating most of the larger, older bivalves in the Chesapeake Bay, USA during 1971-1984 (Holland et al., 1987) and the cockle industry in Bay of Somme, France in 1989 (Desprez et al., 1992). Furthermore, hypoxia caused massive mortality in cockle and oyster aquaculture in Jinhae Bay, Korea in 1978 (Cho, 1991).

As well as the lethal effects of hypoxia, reduced fitness may be observed in individuals exposed to suboptimal oxygen levels. Evidence for reduced fitness may include behavioral changes, reduced feeding rates, decreased aerobic scope, and decreased scope for growth and overall growth (Burnett and

Stickle, 2001). For example, mild hypoxia may cause a reduction in clearance rates in bivalves, such as the clams *Paphies australis* (Norkko et al., 2005) and *Ruditapes decussatus* (Sobral and Widdows, 1997), and the mussel *Mytilus edulis* (Wang and Widdows, 1993b). Aerobic metabolic rates may also be reduced with declining oxygen tension (e.g. *M. edulis* and *Abra tenuis*, Wang and Widdows, 1993a, b). When bivalves are unable to suppress their energy requirements, they must change from aerobic to anaerobic metabolism, which requires them to use their energy reserves to cope with severe hypoxia or anoxia (Grieshaber et al., 1994). Subsequently, their growth is decreased under hypoxic and anoxic conditions (e.g. *Crassostrea virginica,* Baker and Mann, 1992; Breitburg et al., 2015). Hypoxia may also limit the tolerance of bivalves to other environmental challenges, such as temperature (Pörtner, 2010) and disease (Breitburg et al., 2015), or compromise their anti-predator responses (Saloom et al., 2005). For instance, hypoxia/anoxia has been shown to narrow the thermal windows of aerobic scope of intertidal marine bivalves (for a review, see Pörtner, 2010), and hypoxia has been reported to affect negatively the immune system of *C. virginica*, leading to elevated parasite infections (Breitburg et al., 2015). In addition, behavioral responses of bivalves under hypoxia, such as reduced burial depth and extension of siphons, have been shown to increase vulnerability to predation in *Corbicula fluminea* (Saloom et al., 2005) and *Mya arenaria* (Taylor and Eggleston, 2000; Norkko et al., 2013). These threats to production and reductions in animal fitness highlight the importance of understanding both the lethal and sublethal effects of hypoxia on cultivated bivalves.

The New Zealand aquaculture industry has recognized the native New Zealand geoduck clam, *Panopea zelandica*, as an emerging species to contribute to the goal of $1 billion per annum in revenues by 2025 (Carter, 2012). *P. zelandica* is a subtidal species. However, there is a desire by the nascent geoduck aquaculture industry to grow them in intertidal areas, in order to significantly reduce operational costs. Although the current geoduck production is entirely from wild harvest, small-scale farm trials are being conducted. Hatchery-raised juveniles (15 – 20 mm in shell length) are being planted in sediment under mussel and oyster farms as initial pilot trials (K. Heasman, unpublished data). However, there may be trade-offs between operational costs and time-to-harvest or mortality in intertidal areas since geoducks may encounter regular hypoxic conditions that may have negative effects on their growth and survival. The aquaculture production cycle of *P. zelandica* has been estimated to be from 5 to 10 y based on the age-growth relationship of subtidal populations (Breen et al., 1991; Gribben and Creese, 2005). Hence, it is likely that intertidally cultivated geoducks may have even longer production cycles if their growth is reduced under daily hypoxic stress.

To inform husbandry practices, and maximize the growth and production potential of New Zealand geoducks, detailed information is needed about the effects of hypoxia on the growth and fitness of these clams. To study the sublethal effects of hypoxia on the fitness of animals, such as bivalves, aerobic scope and respiratory responses are often used as indicators of stress (Sokolova et al., 2012). In filter-feeding sessile bivalves, the aerobic scope can be determined by the difference between the metabolic rates of fed (routine metabolic rate) and starved (standard metabolic rate) animals (Bayne et al.,

1976). In addition, respiratory responses of bivalves to hypoxic conditions can be quantified by measuring critical oxygen partial pressure (P_cO_2) and regulation index (RI; Hicks and McMahon, 2002; Mueller and Seymour, 2011). Based on respiratory responses, animals are typically classified as oxyconformers or oxyregulators (Newell, 1979). In oxyconformers, oxygen uptake decreases linearly with decreasing oxygen tension (Bayne et al., 1976). Conversely, the response of oxyregulators to declining oxygen availability is to increase water pumping rate or oxygen extraction efficiency in an attempt to maintain the required tissue oxygen levels, and only when oxygen tension decreases to a critical level does oxygen uptake become compromised (Wu, 2002; Le Moullac et al., 2007). The oxygen tension below which oxyregulators cannot maintain their oxygen consumption rates is called the critical oxygen partial pressure (P_cO_2; Grieshaber et al., 1994). This threshold may indicate an abrupt and continuous depression of metabolic rate (Mueller and Seymour, 2011), or reflect a switch from aerobic metabolism to anaerobic metabolism (Pörtner and Grieshaber et al., 1993). However, P_cO_2 has two constraints: i) this indicator cannot be applied to oxyconformers (Pörtner et al., 1985); and ii) it does not provide information as to how bivalves consume oxygen above P_cO_2 (Mueller and Seymour, 2011). Therefore, the regulation index (RI) was additionally introduced to quantify the degree of independence of oxygen consumption on oxygen tension, which can be applied for both oxyregulators and oxyconformers (Hicks and McMahon, 2002; Mueller and Seymour, 2011). The RI is a relative measure of regulatory ability obtained by calculating the area under the oxygen consumption versus oxygen tension curve (Mueller and Seymour, 2011). A perfect oxyregulator would have an RI of 100% since it can

maintain its oxygen consumption rate from normoxia (16.7 - ~21 kPa) to hypoxia (~ 0 - 16.7 kPa); while a strict oxyconformer would have an RI of 50% since its oxygen consumption rate linearly declines with decreasing oxygen tension (Alexander Jr and McMahon, 2004).

Despite generally having fairly high tolerance capacities against hypoxia, the respiratory responses of bivalves are dependent on both exogenous and endogenous conditions (Herreid, 1980; Bayne and Newell, 1983). For example, the capacity to regulate oxygen consumption under hypoxic conditions is reduced when temperatures are not within the optimal physiological range (Bayne and Newell, 1983; Pörtner, 2010). Furthermore, subtidal species, such as the scallop *Pecten maximus*, may display a different pattern of regulating oxygen consumption from intertidal species, such as mussels (*Mytilus* spp.) during hypoxia exposure (Artigaud et al., 2014). Transplantation experiments have demonstrated that acclimation to tidal emersion regime is also responsible for variability in both tolerance to aerial emersion and submerged hypoxia (i.e. *Mytilus edulis*, Altieri, 2006). Moreover, within a species, large animals typically show a better ability to regulate oxygen consumption under hypoxia than small ones (Bayne, 1971a; Taylor and Brand, 1975). Besides exogenous factors, endogenous factors, such as nutritional state (e.g. fed versus starved) also affect oxyregulatory capacity of an organism (Bayne et al., 1976). Thus, there is a consensus that the degree of independence between respiration rates and oxygen tension is associated with the species-specific lifestyle, size, and nutrient state of a given individual.

The present study investigated the sublethal effects of hypoxia on New Zealand geoduck: respiration rates, aerobic scope for activity, critical oxygen

partial pressure levels, and regulation indices for juvenile and adult geoduck under both fed and starved conditions from normoxia to anoxia. It is envisaged that the findings of this work will lead to better-informed cultivation practices of geoduck in coastal areas of New Zealand.

9.2 Materials and methods

9.2.1 Animal husbandry and acclimation

All experimental geoducks were spawned and reared at the Cawthron Institute Aquaculture Park, Nelson, New Zealand. Two size/age classes of animals were used in four separate experiments: small (seven-month-old; 30.63 ± 3.09 mm shell length and 9.04 ± 2.76 g live weight) and large (four-year-old; 63.97 ± 4.99 mm shell length and 115 ± 20.09 g live weight). Small geoducks were individually buried in sand inside 50 ml plastic Falcon™ tubes, while large geoducks were buried in 1000 ml plastic tubes of sand to minimize subsequent handling stress and to provide a stable environment during experiments. Twelve geoducks of each size class were acclimated in flow-through 1 µm filtered seawater at 15°C for 14 days for small-sized individuals and 30 days for large-sized individuals. Half of the animals were starved (six small and six large geoducks), and the other half were *ad libitum* fed *Tisochrysis lutea* (formerly known as *Isochrysis affinis galbana* or *T-ISO clone*; Bendif et al., 2013) at 30,000 – 40,000 cells mL^{-1} by constant feeding of algal cells into the tank via a pneumatic pump. This algal concentration was applied to induce maximal feeding rate based on a pilot study of feeding plateau.

9.2.3 Oxygen consumption measurements

After the acclimation period, the sand tubes containing individual animals were placed in individual respirometry chambers to measure oxygen consumption

over self-induced declining oxygen tensions from normoxia to anoxia, or until the oxygen consumption ceased. Animals were left in the respirometry chambers under flow-through conditions for 1 h before those chambers were closed and oxygen consumption measurements were started. The oxygen levels were continuously recorded over 24 - 25 h for small geoducks and 37 - 67 h for large geoducks in the dark. For small geoducks, chambers consisted of 660 ml acrylic plastic cylinders (60 mm Ø, 5 mm thick) with two Perspex end caps (10 mm thick), submerged in a 15°C water bath. For large geoducks a 7 L glass double-jacketed vessel with a lid sealed with a gasket was used, and the vessel was connected to a water bath to control temperature via the water jacket (Fig. 9.1). The tubes containing the individual geoducks were suspended inside the respirometers, and water was mixed by a magnetic flea beneath to avoid oxygen stratification (Fig. 9.1). Adequate mixing was initially verified using food colouring. Temperature and oxygen sensors (FOXY-R, Ocean Optics, Dunedin, FL) were inserted through the top cap of each chamber, and connected to a phase measurement system (NeoFox®, Ocean Optics) from which signals were recorded every two seconds using NeoFox software. The oxygen sensor was calibrated to 100% and 0% oxygen saturation before each measurement series. Temperature and food conditions were maintained as for the acclimation period for each group. An additional control respiration chamber (containing a tube with sand, but no geoduck) was used to record the background oxygen levels in the system for each group. The potential effect of bacterial and microalgal oxygen consumption was determined to be negligible after the removal of the animals (data not shown).

9.2.4 Respiration rate (RR) and aerobic scope for activity (SFA) calculations

Seawater oxygen content was determined from % saturation after accounting for the effects of temperature, salinity, atmospheric and saturated vapour pressures (after Benson and Krause, 1984; Weiss and Price, 1980). Individual respiration rates (RR; mg O_2 h^{-1}) were then calculated following the equation:

$$RR_e = Slope \times V$$

where RR_e is the respiration rate of the experimental geoduck (mg O_2 h^{-1}), slope is the absolute value of the slope of linear regressions of oxygen concentration over time (mg O_2 L^{-1} h^{-1}), and V is the volume of water in the respiration chamber (L). The slopes were calculated over 1 kPa intervals from 21 kPa to 3 kPa and at 0.2 kPa intervals until 0 kPa or the lowest oxygen tension was reached. The water volume in the chambers was measured after accounting for the volume of the geoduck, sand, tube, holder, and magnetic flea.

At the end of the experiment, the dry weight of geoduck soft tissues were determined by drying at 104°C for 24 h. The respiration rates were standardized to 1 g of tissue dry weight for both small and large geoducks using the formula given by Bayne and Newell (1983):

$$RR = RR_e \times \left(\frac{W_s}{W_e}\right)^b$$

where RR is the standardized respiration rate (mg O_2 g DW^{-1} h^{-1}), RR_e is the measured respiration rate of the experimental geoduck (mg O_2 g DW^{-1} h^{-1}), W_s is 1 g, W_e is the tissue dry weight of the experimental geoduck (g), and *b*, the

allometric exponent for respiration rate of geoducks, which is 0.73 (Le et al. in prep).

Aerobic scope for activity (SFA) was calculated as follows:

$$SFA = RMR - SMR \qquad \text{(Bayne et al., 1976)}$$

where SFA is the aerobic scope for activity, RMR (routine metabolic rate = RR_{fed}) is the standardized respiration rates of fed animals, and SMR (standard metabolic rate = $RR_{starved}$) is the standardized respiration rates of starved animals. All variables have the same unit of mg O_2 g DW^{-1} h^{-1}.

9.2.5 Critical oxygen partial pressure (P_cO_2) and regulation index (RI) calculations

The standardized respiration rates (RR) of each geoduck were divided by the maximum RR over the entire range of oxygen tensions (Hicks and McMahon, 2002) to provide a respiration rate ratio (RR_{ratio}):

$$RR_{ratio} = \frac{RR}{\text{maximum}(RR)}$$

where RR_{ratio} is the respiration rate ratio and RR is the standardized respiration rate (mg O_2 g DW^{-1} h^{-1}).

The aerobic metabolic rates of the entire organism generally follow Michaelis-Menten kinetics in response to oxygen availability:

$$RR_{ratio} = \frac{a \times PO_2}{b + PO_2} \qquad \text{(Hochachka and Somero, 2001)}$$

where RR_{ratio} is the respiration rate ratio, PO_2 is the partial pressure of oxygen (kPa), and a and b are constants (see below).

The critical oxygen partial pressure (P_cO_2) is determined when the slope of the Michaelis-Menten function begins to approach zero according to:

$$P_cO_2 = \sqrt{\frac{ab}{m}} - b \qquad \text{(Marshall et al., 2013)}$$

where P_cO_2 is the critical oxygen level (kPa), a and b are Michaelis-Menten kinetic constants, and m is a slope of 0.05.

The regulation index (RI) was calculated following the equation:

$$RI = \frac{\int_0^{21} RR_{ratio}}{\int_0^{21} 1} \times 100\% \quad \text{(Hicks and McMahon, 2002)}$$

where RI is the regulation index (%), $\int_0^{21} RR_{ratio}$ is the integrated sum of the respiration rate ratio under the Michaelis-Menten curve across 0-21 kPa oxygen tensions.

9.2.6 Statistical analysis

A standard non-linear regression was used to fit the Michaelis-Menten function to the respiration rate ratio. After determining P_cO_2, the standardized respiration rates and aerobic scope for activity of geoducks were pooled into three groups: normoxia (16.7 - ~21 kPa), mild hypoxia (P_cO_2 - 16.7kPa), and severe hypoxia (< P_cO_2). A three-way analysis of variance (ANOVA) was used to analyze the effects of oxygen tension, size class, and feeding condition on the respiration rate of geoducks. A two-way ANOVA was used to determine the effects of oxygen level and size on the aerobic scope of geoducks. The effects of feeding condition and size on P_cO_2 and RI values were tested with a two-way ANOVA. All statistical tests were evaluated at the significance level of 0.05.

P_cO_2 and RI were determined using R software while ANOVAs were run using Minitab v. 17 statistical software.

9.3 Results

9.3.1 Effects of oxygen tension, animal size, and feeding condition on respiration rate (RR)

Oxygen tension, size class and feeding condition (fed or starved) all significantly affected the respiration rate (RR) of geoducks ($P < 0.001$; Table 9.1). In addition, all two-way interactions between size, oxygen tension, and feeding condition significantly influenced the RR of geoducks (Table 9.1). Starved, small geoducks exposed to severe hypoxia showed a RR reduced by 2.5-fold compared to levels under normoxia and mild hypoxia (Fig. 9.2a and c). The RR (mean±SD) of starved small geoducks was 0.37±0.10 mg O_2 g DW^{-1} h^{-1} under normoxia and decreased slightly during mild hypoxia (0.34±0.09), but decreased significantly under severe hypoxia (0.15±0.07; $P < 0.001$; Fig. 9.2c). Fed small geoducks under mild hypoxia and severe hypoxia had a RR which was 1.5- and 2.6-times lower than under normoxia, respectively (Fig. 9.2a and c). The RR (mean±SD) of fed small geoducks declined significantly from 0.53±0.13 mg O_2 g DW^{-1} h^{-1} under normoxia to 0.35±0.09 and 0.20±0.06 under mild and severe hypoxia, respectively ($P < 0.001$; Fig. 9.2c).

As with starved small geoducks, starved large geoducks had the lowest RR under severe hypoxia, 2.3 and 2.1 times lower than in normoxia and mild hypoxia (Fig. 9.2b and d). The RR (mean±SD, mg O_2 g DW^{-1} h^{-1}) of starved large geoducks decreased slightly from normoxia (0.28±0.07) to mild hypoxia (0.25±0.04) and decreased significantly under severe hypoxia (0.12±0.07; $P < 0.001$; Fig. 9.2d). The RR (mean±SD, mg O_2 g DW^{-1} h^{-1}) of fed large geoducks

did not change from normoxia (0.33±0.09) to mild hypoxia (0.34±0.07) but decreased significantly under severe hypoxia (0.20±0.10; $P < 0.001$; Fig. 9.2d).

9.3.2 Effects of oxygen tension and size on scope for activity (SFA)

Scope for activity is the difference between fed (active) metabolic rate and starved (standard) metabolic rate. Under normoxia small geoduck had a mean (±SD) SFA of 0.2±0.1 (mg O_2 g DW^{-1} h^{-1}), and large geoduck had a four-fold lower SFA of 0.05±0.02. Oxygen tension had a significant effect on the SFA of geoducks ($P = 0.004$) but size alone had no significant effect on SFA ($P = 0.6$; Table 9.2). However, there was a significant interaction effect between oxygen tension and size ($P < 0.001$; Table 9.2), indicating that different sizes were differentially affected by changes in oxygen tension. The SFA of small geoducks decreased significantly under mild and severe hypoxia (from 0.2±0.1 mg O_2 g DW^{-1} h^{-1}, mean±SD) under normoxia to 0.01±0.08, and 0.05±0.04, under mild and severe hypoxia respectively; $P = 0.001$; Fig. 9.2e). In contrast, there was no significant effect of oxygen tension on SFA of large geoducks, with a slight increase observed from normoxia (0.05±0.02 mg O_2 g DW^{-1} h^{-1}, mean±SD) to mild and severe hypoxia (0.09±0.02 and 0.08±0.05, respectively; $P = 0.198$; Fig. 9.2f).

9.3.3 Effects of size and feeding condition on critical oxygen partial pressure (P_cO_2) and respiration index (RI)

There was no significant effect of either size ($P = 0.758$) or feeding condition ($P = 0.266$) on the critical oxygen partial pressure (P_cO_2) of geoducks (Table 9.3). The (mean±SD) P_cO_2 of starved and fed small geoducks were 4.72±1.62 and 4.19±2.92 kPa, respectively, and in large geoducks P_cO_2 were 4.85±1.89 and 3.57±0.68 kPa, respectively (Fig. 9.3).

Size had a significant effect on the regulation index (RI) of geoducks (P = 0.013; Table 9.4). Large geoducks had a significantly better capacity to regulate oxygen consumption under hypoxia than small ones with an RI (mean±SD) in large geoducks of 68.31±9.24% compared to 55.67±12.19% for small geoducks (Fig. 9.3). However, feeding condition did not affect RI (P = 0.651) nor was there any interaction between size and feeding condition (P = 0.412) (Table 9.4). The RI (mean±SD) of starved large geoducks (65.32±10.92%) was not significantly different from that of fed large individuals (71.30±6.86%), and the RI of starved small geoducks (56.47±14.23%) was not significantly different to that of fed small geoducks (54.72±10.77%).

9.4 Discussion

9.4.1 Effect of PO_2, size, and feeding on RR

The findings of this study indicate that oxygen tension, size class, and feeding status all affected the respiration rate (RR) in geoducks. The standardised RRs of small geoducks were significantly higher than those of large geoducks were across all oxygen tensions and in both starved and fed animals (Fig. 9.2). Similarly, the RRs of small mussel *M. edulis* (Bayne, 1971) and small clam *Arctica islandica* (Taylor and Brand, 1975) were higher than those of larger individuals were. It is well-known that small (young) individual bivalves have higher specific growth rate (growth increment by day) than larger (older) individuals (e.g. Gribben and Creese, 2005). Since respiration and growth are frequently correlated, small (young) individuals usually show higher weight specific RRs than larger (older) individuals (Bayne et al., 1976). This size-specific difference in RR is in agreement with the allometric scaling principle

that mass-specific metabolic rate decreases with increasing body mass (Bougrier et al., 1995; Karasov and Martínez del Rio, 2007).

The results also show that RR of fed geoducks was higher than those of starved individuals within all oxygen tension treatments and geoduck size classes. Similar results were obtained for other fed and starved bivalves, such as the mussel *M. edulis* (Bayne et al., 1976; Wang and Widdows, 1993b) and the clam *Ruditapes philippinarum* (Tamayo et al., 2013). In the absence of food, the RR of bivalves declines to a steady-state condition in which filtration activity is minimal, as a result, the energy is only utilized for maintenance. In contrast, when such an animal is fed, filtration rate increases, with a disproportionate increase in RR, reflecting the metabolic cost of other processes, such as digestion, assimilation, egestion, excretion, and growth. These results are in agreement with the universal theory that RR of fed or exercised animals (known as routine metabolic rate, RMR) is higher than that of starved or resting individuals (known as standard metabolic rate, SMR) (Fry, 1947; Bayne et al., 1976; Pörtner, 2010).

Beside their isolated effects, oxygen tension, size and feeding condition also had interactive effects on RR of geoducks. The SMR of small geoducks was maintained from normoxia to mild hypoxia while the RMR of small geoducks decreased significantly. Similarly, the RMR of small *M. edulis* reduced within mild hypoxic conditions and their SMR were maintained when being exposed to the same conditions (Wang and Widdows, 1993b). Unlike small individuals, both SMR and RMR of large geoducks were maintained from normoxia to mild hypoxia. Our current findings are in agreement with the maintenance of both RMR and SMR in large mussels, *M. edulis*, and clams,

Abra tenuis, experiencing mild hypoxia (Wang and Widdows, 1993a, b). The surface of the mantle cavity was the predominant respiratory site in the mussel *M. edulis*, and probably other bivavles (Famme and Kofoed, 1980; Jørgensen et al., 1986). Hence, it is suggested that large bivalves' respiration is increasingly dependent upon the ability of the mantle cavity to uptake oxygen rather than the gills. The mantle cavity plays a role as a diffusion chamber from which oxygen is taken up by the arterial haemolymph. However, instead of being normoxic, the oxygen tension in this chamber is always at a severely hypoxic level for large bivalves (< 8kPa), even when animals are kept under normoxic conditions (Abele et al., 2010). It has been suggested that the PO_2 in the mantle cavity of bivalves is kept low enough to protect haemolymph and tissues against overoxygenation and to avoid increased formation of hazardous reactive oxygen species, and high enough to support effective diffusion (Abele et al., 2010). Hence, external mild hypoxic conditions are less likely to cause oxygen deficiency in the mantle cavity of large bivalves, reducing the potential impact on their RR. This result is consistent with observations that the respiration of larger gastropod molluscs is relatively independent of oxygen tension during mild hypoxia (Marsden et al., 2012).

However, in the present study, when PO_2 was lower than P_cO_2, both SMR and RMR of geoducks decreased significantly regardless of size. This result is a symptom of the failure of oxyregulation, observed in both fish and invertebrates when PO_2 is below P_cO_2 (Wu, 2002). However, while the failure of oxyregulation in fish may coincide with the onset of anaerobiosis (Pörtner and Grieshaber, 1993; Pörtner, 2010), bivalves may depress their aerobic metabolism and enter a hypometabolic state, instead of switching to anaerobic

metabolism (Wang and Widdows, 1993a, b; Sobral and Widdows, 1997; Clark et al., 2013). For example, the intertidal clam *Abra tenuis* and the intertidal mussel *M. edulis* were shown to decrease their aerobic metabolism when PO_2 was below P_cO_2, 5 kPa, but the onset of anaerobic metabolism only occurred when PO_2 was below 2.3 kPa (Wang and Widdows, 1993a, b). Rather than utilize the energy reserve for anaerobic metabolism, the subtidal clam *Ruditapes decussatus* slightly opened their valves periodically and depressed their aerobic metabolic rates until anoxic (0 kPa; Sobral and Widdows, 1997). Despite significantly decreasing respiration rates when oxygen tension was below P_cO_2 (~ 4 kPa), *P. zelandica* periodically opened its siphons, suggesting that geoducks may be able to enter a hypometabolic state during severe hypoxia and even anoxia. Other subtidal deep burrowing clams, such as *A. islandica* and *Laternula elliptica* have also been suggested to exhibit hypometabolism (Strahl et al., 2011; Clark et al., 2013). Indeed, metabolite analysis of tissues showed no increase in intermediate (i.e., nicotinamide adenine dinucleotide) or end-products (i.e., octopine) of the anaerobic metabolism pathway under long-term severe hypoxia in clams (Strahl et al., 2011; Clark et al., 2013). Hence, bivalves tend to suppress their metabolism, and accordingly decrease their aerobic scope and growth during severe hypoxia. This sublethal effect of hypoxia is seen in not only subtidal but also intertidal bivalves at both individual and population levels (i.e. *M. edulis*, Altieri and Witman, 2006).

9.4.2 Effect of PO_2 and size on SFA

This study showed that SFA in geoducks was affected by the interaction between oxygen tension and size. Small geoducks (~30 mm shell length, 9 g

live mass) had higher SFA than large geoducks (~64 mm, 115 g) within normoxia, which may be indicative of higher scope for growth or faster net growth in small geoducks. These size-specific SFA and growth patterns within normoxia have been observed in other bivalves. For example, Philipp et al. (2008) showed that small scallops, *Aequipecten opercularis*, had higher SFA than large individuals within normoxia, while the scope for growth was higher in small pearl oysters (*Pinctada maxima* and *P. margaritifera*) than in large oysters under ambient conditions (Yukihira et al., 1998). The reduction in SFA with increasing size provides insight into why the growth rate of geoducks, and other bivalves, is generally slower after the first few years (Gosling, 2003; Gribben and Creese, 2005; Gilbert et al., 2014; Peck et al., 2007).

However, in the present study, when geoducks were exposed to hypoxia, SFA of small individuals decreased, suggesting a depression in active (routine) metabolism of small geoducks. This metabolic depression strategy has also been reported in small mussels, *M. edulis* (Wang and Widdows, 1993b), *Perna viridis* (Wang et al., 2010), small clams, *Paphies australis* (Norkko et al., 2005), *R. decussatus* (Sobral and Widdows, 1997), and small oysters, *C. virginica* (Baker and Mann, 1992). Under stress conditions, such as hypoxia, energetic investment is prioritized differently at different stages of the life cycle (Sokolova, 2013). Smaller invertebrates frequently utilize the metabolic depression strategy in which animals conserve energy necessary to carry out physiological processes in stress conditions (Grieshaber et al., 1994), therefore, extending their survival window (Sokolova, 2013). However, the trade-off of metabolic depression is the reduction in SFA, which might result in a decrease in scope for growth and actual size gain (Sokolova et al., 2012). Hence, the reduction in

SFA in mild hypoxia may cause negative effects on the growth of small geoducks.

Conversely, in this study, SFA in large geoducks did not decrease under mild and severe hypoxia, suggesting a lack of capacity to utilise metabolic compensation in large geoducks. Similarly, large *L. elliptica* did not reduce metabolic rates or conserve energy reserves under hypoxia (Clark et al., 2013). Increasing SFA rather than suppressing metabolism has been suggested as a mechanism for large bivalves to compensate for the oxygen deficiency (Peck et al., 2007). However, if the oxygen supply decreased isometrically with increasing body size, large individuals would be less efficient at delivering oxygen to their mitochondria (Atkinson et al., 2006). Indeed, prolonged severe hypoxia caused mortality of large individuals prior to that of small individuals in various long-lived clams, such as *L. elliptica* (Clark et al., 2013), and *Mya arenaria* and *Macoma balthica* (Norkko et al., 2013; Villnäs et al., 2012). Hence, the present study is consistent with previous research indicating that larger bivalves are better able to maintain their aerobic scope and, presumably, activity levels under mild hypoxia. As this strategy may be associated with a survival trade-off if hypoxic conditions are maintained, further research should focus on the interaction between energetic compensation strategies and survival of large geoducks under prolonged, severe hypoxia.

9.4.3 Effect of size and feeding status on P_cO_2

All geoduck groups shared a similar P_cO_2 (~ 4 kPa) regardless of size and feeding status (well fed or fasting). The similar P_cO_2 between two size classes in geoducks agrees with the results of Wang and Widdows (1993b) in which small and large *M. edulis* had a similar re-calculated P_cO_2 mean, 6-7 kPa

(values are re-calculated using the nonlinear regression approach). In contrast, the re-calculated P_cO_2 mean of small *A. islandica* (~ 6 kPa) was higher than that of larger individuals (~ 4 kPa, Taylor and Brand, 1975). In addition, the similar P_cO_2 between starved and fed geoduck provides compelling evidence that nutritional stress does not affect P_cO_2 in geoducks. Similarly, nutritional stress did not affect re-calculated P_cO_2 in *M. edulis* (6-7 kPa; Wang and Widdows, 1993b) and *Abra tenuis* (~ 6 kPa; Wang and Widdows, 1993a). Since P_cO_2 appears unaffected by either size or feeding status in most of observed bivalve species, P_cO_2 in bivalves may only indicate the abrupt change in their respiration rate, but not reliably identify the limits of oxygen regulation capacity, as proposed in previous studies (Hicks and McMahon, 2002; Mueller and Seymour, 2011).

9.4.4 Effect of size and feeding status on RI

Our results indicate that size rather than feeding status affected overall oxyregulatory capacity in geoducks, as measured by the regulation index (RI). Large geoducks (~10.8 g DW) had significantly higher capacity than small individuals (0.58 g DW) to regulate oxygen consumption during hypoxia (Fig. 9.3). The same effect of size on RI has been found in other infaunal bivalves, including *Laevicardium crassurn* (0.16 – 3.14 g DW, Bayne, 1971a), *A. islandica* (0.03 – 1 g DW, Bayne, 1971a; 2.9 – 16 g DW, Taylor and Brand, 1975), and *Mulinia lateralis* (< 5 mm to > 10 mm shell length, Shumway, 1983). Similarly, larger epifaunal bivalves had higher RI than smaller individuals, including *Perna perna* (0.4 – 3.0 g DW, Bayne, 1967) and *M. edulis* (0.34 – 1.27 g DW) (Bayne, 1971a). As direct oxygen uptake via the body surface (Schmidt-Nielsen, 1975) can account for a relatively large proportion of total oxygen uptake in bivalves,

such as 85 – 100% in mussels (*Geukensia demissa*, Booth and Mangum, 1978; *M. edulis*, Famme, 1981), increasing body size might be an advantage to regulate oxygen consumption under hypoxia. Indeed, biochemical evidence of oxygen uptake via the body surface was recently found in siphon and mantle tissues of the clam *Laternula elliptica* under hypoxia (Clark et al., 2013), and results also suggested that siphon and mantle tissues were more hypoxia sensitive than gill tissues. Hence, larger surface area of siphon and mantle in large *P. zelandica* may account for their greater oxygen regulation capacity. Also, diffusion limitation and surface area relationships were suggested to be reasons for the better oxygen regulation capacity in larger individuals for other molluscs, including gastropods (Marsden et al., 2012). Further work is required to elucidate the effects of changing allometry of diffusion surfaces and the effects of increasing siphon size upon ventilatory flow and net oxygen uptake.

In contrast to size, in this study, nutritional stress (fasting) did not influence RI in geoducks. Starved and fed geoducks had a similar oxygen regulation capacity. Similarly, there was no difference in oxygen regulation capacity between fed and starved *M. edulis* (Wang and Widdows, 1993b) and *Perna perna* (Bayne, 1967; Hicks and McMahon, 2002). Conversely, Bayne (1971b) found a failure of oxygen regulation due to nutritional stress in *M. edulis*. These apparently contradictory results may be due to the difference in salinity, which was lower (18 ppt) in Bayne (1971b) than in other studies (> 30 ppt, Bayne, 1967; Wang and Widdows, 1993b; Hicks and McMahon, 2002). Since bivalves decrease their respiration rate with decreasing oxygen tension and salinity (e.g. *Perna viridis*, Wang et al., 2011) or close their shells (e.g. *Anadara granosa*, Davenport and Wong, 1986), they may lose the oxygen

regulation capacity. Although there is a variation in the effects of fasting on the oxygen regulation among bivalves, most show poor (60-70% RI) to moderate oxygen regulatory capacity (70-80% RI), regardless of feeding condition (Hicks and McMahon, 2002). This poor to moderate oxygen regulation capacity under hypoxia may represent the intrinsic capacity to reduce the metabolic rate of bivalves, rather than rely upon their anaerobic capacity, which may contribute to their profound tolerance of hypoxia (Massabuau and Abele, 2012).

9.4.5 Aquaculture context

One of the principal concerns when establishing an aquaculture farm is to choose a site that is cost-effective in terms of operation and cultivation. Operation costs include monitoring, husbandry, and harvesting activities, while cultivation costs are affected by growth efficiency, growth rate and production cycle. Two of the most promising places to grow geoducks in New Zealand are subtidally under mussel farms or within intertidal oyster farms. It is clear that if hypoxia constrains an animal's growth, the environmental hypoxia associated with periodic emersion in intertidal areas (Pörtner et al., 1984) may compromise productivity and increase cultivation costs. The coastal hypoxia as a result from the interactions of natural and anthropogenic processes, which cause bottom-water hypoxic (Middelburg and Levin, 2009), should also be considered when determining site suitability for geoduck grow-out. Thus, the findings of the present study suggest that the effect of hypoxia on the respiratory performance and aerobic scope of geoducks is a critical factor to account for when determining cultivation costs. The results suggest that small geoducks are likely to experience reduced growth and, potentially, overall fitness during both mild and severe hypoxia due to the decrease in their aerobic scope. Hence,

intertidal environments are likely to be sub-optimal for small *P. zelandica*. On the other hand, large *P. zelandica* may maintain their growth during mild hypoxia, but may experience mortality under prolonged, severely hypoxic conditions, as they attempt to maintain their high aerobic scope. Therefore, it may be possible to grow large geoducks in lower intertidal areas where emersion, and associated hypoxia, is limited. Besides the effect of hypoxia on respiration and aerobic scope, it is worth noting that hypoxia could also change the behaviour of bivalves, potentially increasing vulnerability to predators. While epifaunal bivalves respond to hypoxia by closing their valves (Ortmann and Grieshaber, 2003), infaunal bivalves either reduce the burial depth (Long et al., 2008) or extend their siphons over the sediment surface (Strahl et al., 2011). We also observed that geoducks extended their siphons under hypoxia. Hence, their siphons are at high risk of attack by predators, including sea star, gastropods, crab, and fish (Feldman et al., 2004).

In summary, land-derived sources of eutrophication are expected to continue to increase, resulting in the expansion of hypoxic regions. The acute effects of hypoxia may result in massive mortality of burrowing bivalves (Baden et al., 1990; Norkko et al., 2013). However, the insidious sublethal effects of hypoxia are physiological stress and growth reduction (Allison et al., 2011). From an aquaculture perspective, to avoid stress and maximize the growth potential of geoducks, the results of the present study suggest that a good strategy may be to grow younger, smaller geoducks in subtidal, normoxic areas and transfer large individuals to either subtidal or intertidal farms that experience short periods of mild hypoxia. From an ecological point of view, more research is needed on the hypoxic tolerance capacity of long-lived

species, such as geoducks, as losses may have a significant economic impact on the aquaculture industry, as well as on the sustainability of wild populations (Norkko et al., 2013; Clark et al., 2013).

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Table 9.1 The effect of oxygen tension (PO_2), size class and feeding condition (Feed) on respiration rates of geoducks (Three-way ANOVA). Bold figures identify significant effects ($P < 0.05$).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
PO_2	2	3.02192	1.51096	214.22	**<0.001**
Size	1	0.48613	0.48613	68.92	**<0.001**
Feed	1	0.53457	0.53457	75.79	**<0.001**
PO_2*Size	2	0.25395	0.12697	18	**<0.001**
PO_2*Feed	2	0.05102	0.02551	3.62	**0.028**
Size*Feed	1	0.00031	0.00031	0.04	0.835
PO_2*Size*Feed	2	0.15177	0.07589	10.76	**<0.001**
Error	440	3.10347	0.00705		
Total	451	7.40787			

Table 9.2 The effect of oxygen tension levels (PO_2) and size on aerobic scope of geoducks (Two-way ANOVA). Bold figures identify significant effects ($P < 0.05$).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
PO_2	2	0.039125	0.019563	6.4	**0.004**
Size	1	0.000714	0.000714	0.23	0.631
PO_2*Size	2	0.086621	0.04331	14.16	**<0.001**
Error	43	0.131486	0.003058		
Total	48	0.258288			

Table 9.3 The effect of size and feeding condition (Feed) on critical oxygen partial pressure (Two-way ANOVA).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Size	1	0.3457	0.3457	0.1	0.758
Feed	1	4.6529	4.6529	1.31	0.266
Size*Feed	1	0.8149	0.8149	0.23	0.637
Error	19	67.3905	3.5469		
Total	22	73.4745			

Table 9.4 The effect of size and feeding condition (Feed) on regulation index (Two-way ANOVA). Bold figures identify significant effects (P <0.05).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Size	1	923.84	923.84	7.6	**0.013**
Feed	1	25.64	25.64	0.21	0.651
Size*Feed	1	85.36	85.36	0.7	0.412
Error	19	2308.57	121.5		
Total	22	3340.59			

Figure 9.1 Experimental respirometer setup showing the respiration chamber with geoduck inside, oxygen and temperature probes and measuring instrumentation.

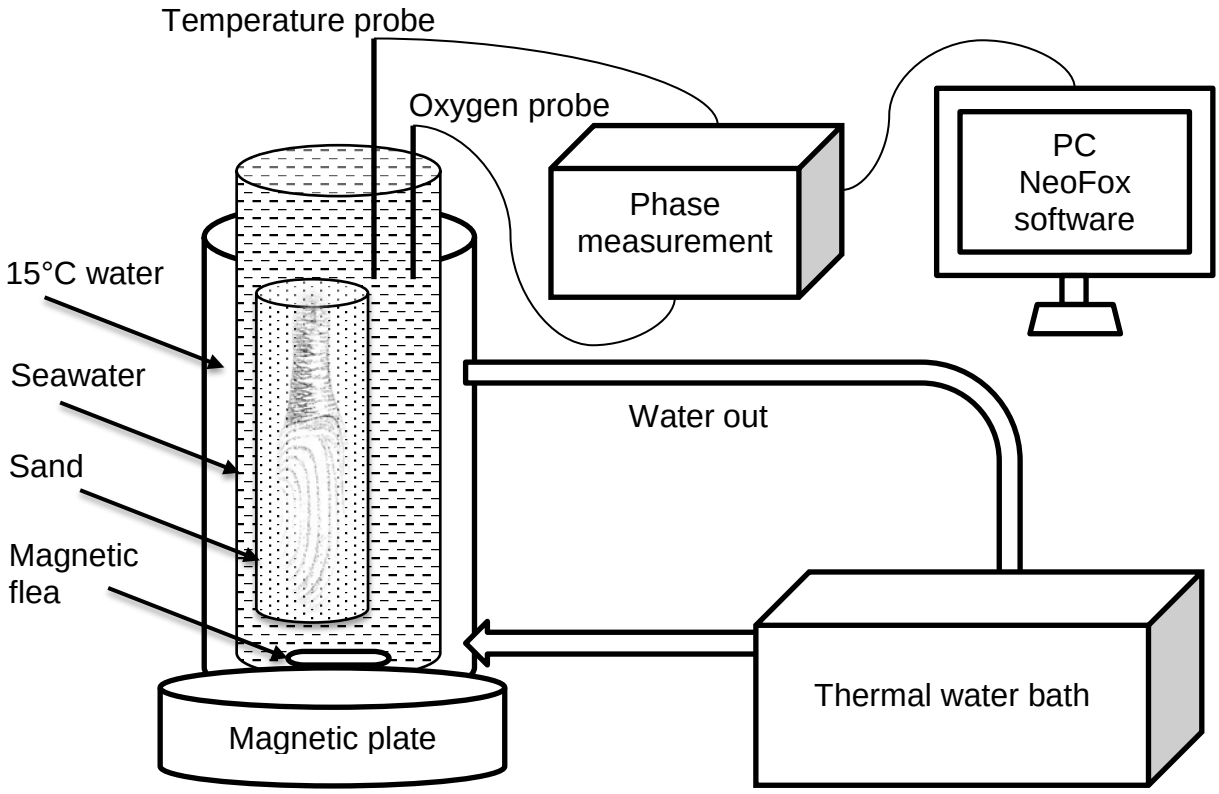


Figure 9.2 Respiration rates (mg O_2 g DW^{-1} h^{-1}) of a) small and b) large *P. zelandica* under starved and fed conditions in relation to oxygen tension PO_2 (kPa), with corresponding mean values under normoxia, mild hypoxia and severe hypoxia summarised in c) and d). Aerobic scope (mg O_2 g DW^{-1} h^{-1}) of e) small and f) large *P. zelandica* in response to normoxia, mild hypoxia and severe hypoxia. All data shown are mean±SD; n = 6. SS represents starved small, FS: fed small, SL: starved large and FL: fed large geoducks. Distinct lower case letters and upper case letters indicate significant differences in mean values of starved and fed geoducks, respectively, between oxygen tensions (P<0.05).

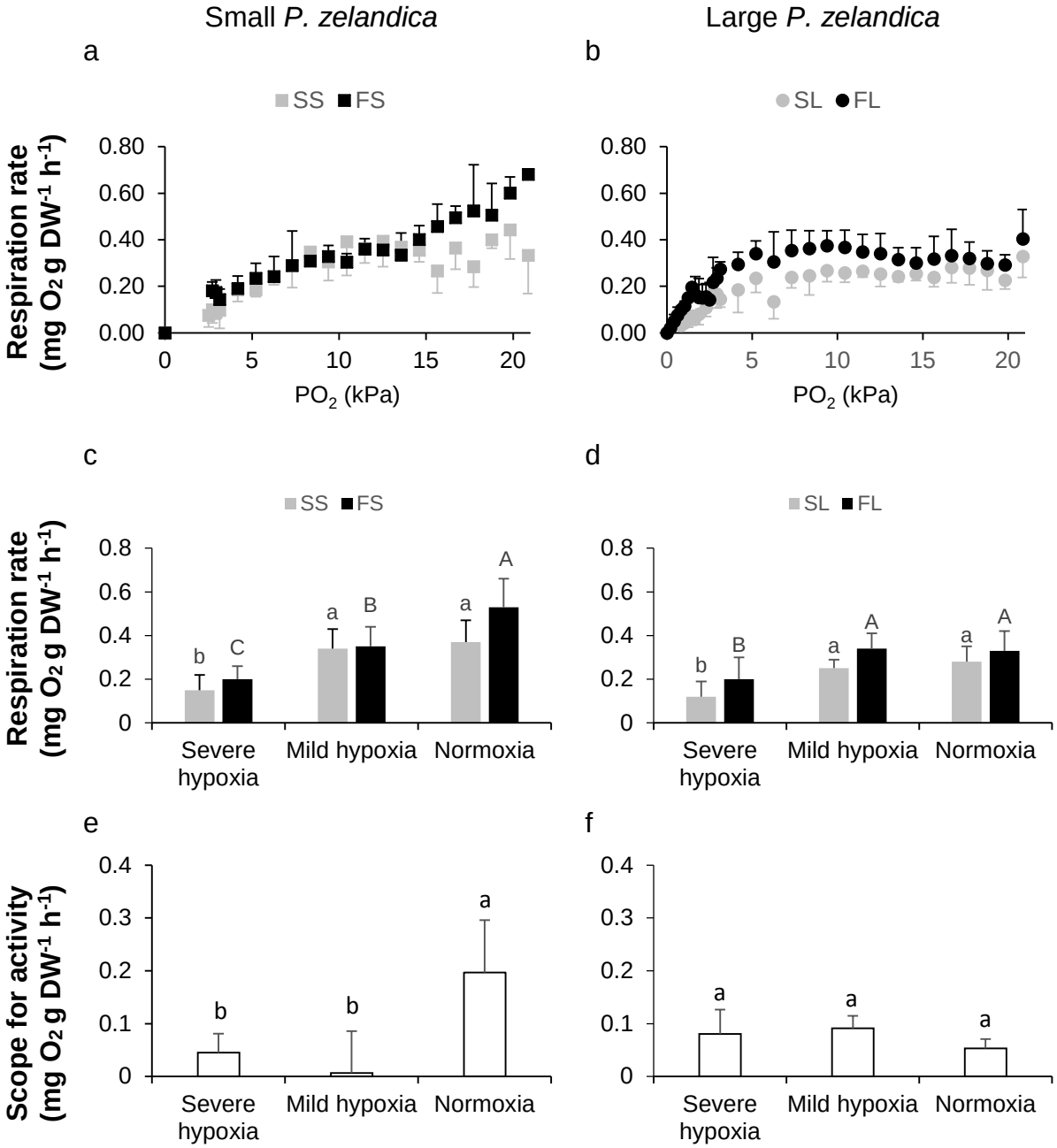
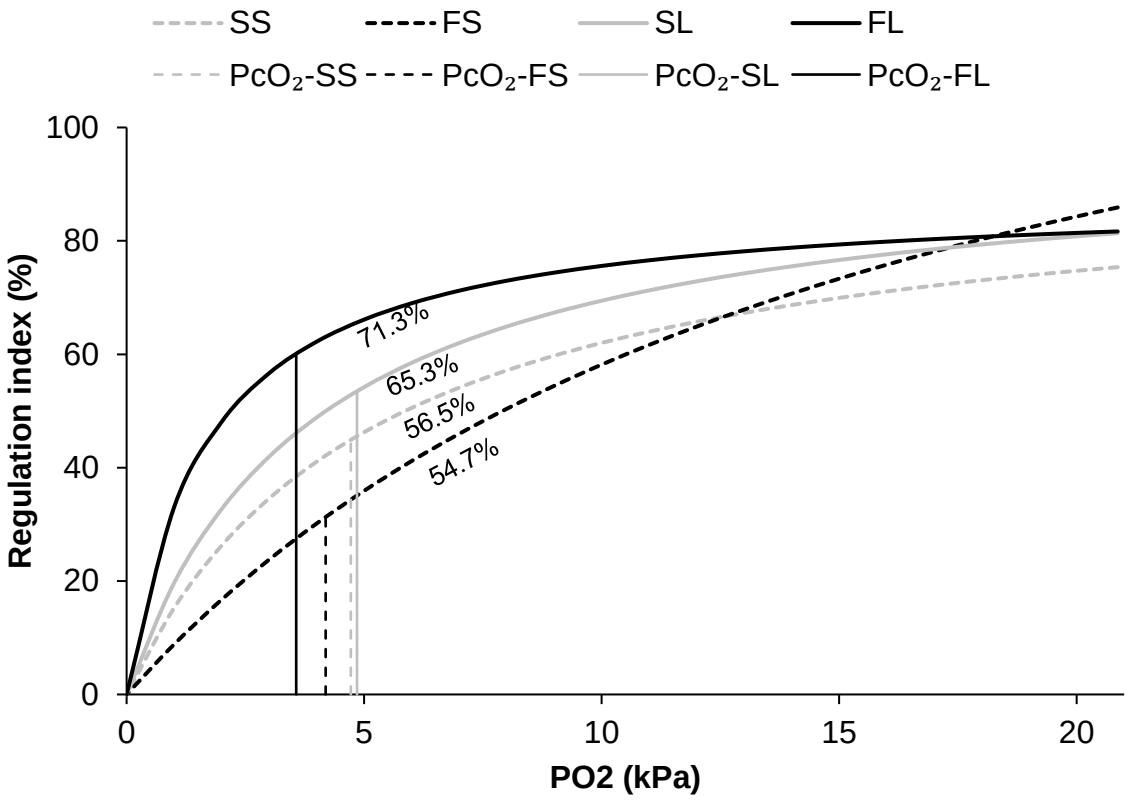

Small P. zelandica
Large P. zelandica
a
b
c
d
e
f
SS FS
SL FL
Respiration rate (mg O2 g DW-1 h-1)
Scope for activity (mg O2 g DW-1 h-1)
PO2 (kPa)
Severe hypoxia
Mild hypoxia
Normoxia

Figure 9.3 Regulation index (%) and critical oxygen level (P_cO_2) of small and large *P. zelandica* under starved and fed conditions, subjected to declining in oxygen tension PO_2 (kPa). The integrated areas under the curves represent the regulation index. The vertical lines represent P_cO_2. SS corresponds to starved small, FS to fed small, SL to starved large, and FL to fed large geoducks. Mean±SD; n = 6.



CHAPTER 10 – Discussion and Conclusion

10.1 Thesis background

“Captain Nemo pointed to this prodigious heap of shellfish, and I saw that these mines were genuinely inexhaustible, since nature's creative powers are greater than man's destructive instincts.”

— Professor Pierre Aronnax (Twenty Thousand Leagues Under the Sea by Jules Verne)

The creation of the submarine became real. However, shellfish resources are not inexhaustible as Professor Aronnax thought. The increasing population and food demands impose vicissitudes on the ‘shellfish mines’. In addition, the advances of fisheries technology lead man’s destructive instincts to become greater than nature’s recruitment rate. Hence, rather than continuing to hunt in the sea we should invest in farming the oceans so they continue supplying all our wants.

The potential for geoduck aquaculture in New Zealand has been recognized from three studies of Gribben et al. during the 1990s, which were published later on (Gribben and Hay, 2003; Gribben et al., 2004; Gribben et al., 2014). Unfortunately, since then, no further studies have been conducted on hatchery techniques and farming conditions. Geoduck aquaculture has only re-gained attention recently when it was identified as a new target aquaculture species, to contribute to New Zealand’s goal of NZD$1 billion aquaculture export value by 2025 (Carter, 2012). Thus, this study was conducted in a collaboration between the Cawthron Institute and the Auckland University of Technology to facilitate the establishment of geoduck aquaculture.

This study presents investigations on the biology, biochemistry, and physiology of the New Zealand geoduck, *Panopea zelandica* reared in the hatchery. *P. zelandica* adults were exposed to different temperature and feeding regimes to determine optimal levels for broodstock conditioning (i.e. reproductive development and nutrient state). Different sperm:egg ratios were tested to identify the optimal ratio and feasible practices for gamete fertilization for hatchery operators. The growth, survival, and ingestion rates of different larval rearing batches were reported to evaluate reasonable practices in terms of stocking density and feeding regime. The functional morphological development of embryos and larvae were described to enhance the hatchery operator's ability to assess larval health and performance. The inducing effects of different neuroactive compounds on larval metamorphosis were tested to improve spat yield. The physiological responses of juveniles at different temperature and oxygen levels were investigated to enable appropriate site selection for juvenile out-growing. Similarly, physiological responses of adults at various temperature and oxygen levels were measured to determine suitable sites for long-term grow-out. The findings in this study provide critical information on conditions and practices to produce *P. zelandica* seed and select farm sites. It is envisaged that this information will form the foundation for geoduck hatchery practices, as well as their farming, to develop a successful New Zealand geoduck aquaculture industry.

10.2 Discussion

"What came first, the chicken or the egg?"

Aristotle – a philosopher and scientist

The same question can be asked for bivalves, what came first, the benthic or the planktonic. Similarly, for geo-duck, what came first, the 'geo' or the 'anatine'? This very simple question has not had a definitive answer. However, whatever the answer is, there is a consensus that the egg must come from the matured broodstock. This study is the first report to date on conditioning geoduck broodstock from the immature stage (spent/resorbed, early active) to the mature stage in hatchery conditions (Chapter 2 and 3). Unlike the geoduck aquaculture industry in USA and Canada, the availability of New Zealand geoduck broodstock is low due to the estimated low densities and small population sizes (Gribben and Heasman, 2015). Hence, instead of collecting ripe wild broodstock every production season, *P. zelandica* broodstock must be kept in hatchery conditions and conditioned from the immature stage. Based on previous investigations of the reproductive development of wild *P. zelandica* (Gribben et al., 2004) and hatchery *P. generosa* (Marshall et al., 2012), a feasible range of temperatures and feeding regimes was determined for studying *P. zelandica* broodstock conditioning (Chapter 2 and 3). A broodstock conditioning guideline for *P. zelandica* was established based on the results of these studies. Briefly, the broodstock conditioning process may be divided into two phases (Chapter 2). When geoducks have yet to start gametogenesis in winter (June-August), they may be provided pond algae at a ration of 50,000 cells mL^{-1} in a flow-through system (Phase I). This pond-nursery system includes land-based seawater ponds, which are enriched with nutrients to promote diatom growth and maintained on multi-week rotations with regular *ad hoc* top up of fresh seawater. The objective of Phase I is to boost the levels of nutrient reserves that will later be mobilized to gamete development. After 4 to

6 weeks of Phase I, *P. zelandica* broodstock may be transferred into controlled conditions where temperature is gradually increased or decreased (1 to 2°C day^{-1}) to either 8 or 12°C and a mixture of *Tisochrysis galbana* and *Chaetocerous muelleri* (1:1 cell counts) provided at a ration of 50,000 cells mL^{-1}. The objective of Phase II is to trigger the gametogenesis process. The high levels of arachidonic acid (ARA, C20:4n-6) in *C. muelleri* may be the cue for triggering this process (Chapter 3). Also, eicosapentaenoic acids (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) appeared to be important fatty acids for broodstock conditioning and gametogenic development of *P. zelandica*. This approach has been shown to maintain a positive energy balance for long-term conditioning of *P. zelandica* broodstock unless the animals are exposed to the extreme conditions of high temperature (16-17°C) and low food ration (10,000 cells mL^{-1}) (Chapter 3). The success of conditioning 2-year old geoducks as a broodstock source will also shorten generation times in any future selective breeding programme. Further research may focus on developing non-invasive methods (e.g. magnetic resonance imaging, endoscopy, vitellogenin in haemolymph) to determine sex and gonad stage of *P. zelandica*.

"Perhaps too much of everything is as bad as too little."

Edna Ferber – a novelist

After obtaining mature broodstock, the second step in hatchery production is to collect gametes and fertilize eggs with sperm. Either too little or too much sperm will cause the fertilization to be incomplete (Bode and Marshall, 2007);

hence, it is crucial to determine the right ratio of sperm and egg. After Gribben et al. (2014), this study is the second report on fertilization for *P. zelandica*. Since the suggested gamete age and contact time by Gribben et al. (2014) may not be the most practical of conditions to conduct a commercial-scale fertilization run, this study re-examined the sperm:egg ratio for optimal fertilization for *P. zelandica*. The present study focused on practical aspects considering the available facility and operational feasibility for fertilization. Also, the embryonic development was described. The result of 97% normal embryos (over the total number of eggs) in this study was expected and was used to establish feasible fertilization practices in the hatchery (Chapter 4). In brief, *P. zelandica* broodstock may be induced to spawn with serotonin injection. Gametes were collected separately every 0.5 h. Eggs and sperm can be stored at 4°C for up to 1.5 and 4 h, respectively. Sperm can be mixed to the egg solution at the ratio of 50-500:1 for 40 min. When the first polar body is observed in most of the eggs, excessive sperm can be removed by rinsing the egg solution through a 45 µm mesh. Zygotes are then incubated in 1 µm filtered seawater and 4 µmol EDTA at 17°C. The use of cold storage may achieve a compromise between the low viability of 'old' egg age and the polyspermic susceptibility of newly serotonin-spawned eggs. Furthermore, cold storage may add flexibility to spawning and fertilization times for hatchery operators since inevitably, under commercial operation, eggs need to be pooled until sufficient numbers have been collected to stock an incubation tank, which may take several hours. The high fertilization rate can also allow triploidy induction to be more efficient. Hence, further research may need to confirm the polyspermic susceptibility of *P. zelandica* serotonin-spawned eggs and to

determine the extent to which cold storage can prolong gamete viability and the mechanisms underlying the viability of geoduck gametes at low temperatures.

"Creating environments which nurture development"

Peter G. Taylor (1997) – a lecturer and researcher

After 48 h embryo incubation at 17°C, D-larvae are formed. New D-larvae enter a mixotrophic phase in which they utilize simultaneously yolk reserves and planktonic particles. Subsequently, D-larvae are in an exotrophic phase in which they rely on only planktonic particles, whereas organogenesis occurs substantially within a short period of time and the vast majority of larvae usually cannot survive through this stage. Hence, for the 'early bird to catch the worm', the more we understand about organogenesis and organ functionality within *P. zelandica* larvae, the better environments we may create to nurture larval development. The description of larval development by Gribben and Hay (2003) illustrated the potential of producing *P. zelandica* spat. Within the past 20 years, larval rearing technology has been developed for *Perna canaliculus* and *Crassostrea gigas* at the Cawthron Institute with the merit of a modern flow-through system. This ultra-high density larval system, which is custom-designed, requires not only large quantities of high quality algae, but also a good understanding of the operation. Thus, it is quite clear that there is a need to determine the rearing conditions for *P. zelandica* larvae in this flow-through system. Hence, this study reported survival, growth, and ingestion rate of several larval batches and general guidelines for applicable practices (Chapter 5). Also, larval organogenesis was linked with organ function and performance

throughout the larval development. The practice which achieved the survival of 76% and the growth rate of 15 µm day^{-1} will be used as a standard operating procedure until further improvement is made. This survival is among the highest reported values for larval rearing across bivalve species, especially at such high density. Also, the organogenesis description in this study represents the first morphological and functional report that integrates systematic, developmental, behavioural, and physiological aspects for geoducks. In short, *P. zelandica* larvae are reared in the flow-through system (see King et al., 2005) with 1 µm FSW at 17±1°C and 35 ppt. The initial stocking density is was 100 larvae mL^{-1}. The geoduck larvae are fed continuously with *T. lutea* and *C. calcitrans,* and the algal residual background level is maintained at 20,000 cells mL^{-1}. Since *P. zelandica* larvae can grow and survive well at the low feeding level, and the algal costs may be reduced significantly in commercial operation. Also, the results on growth and survival suggest that the operation efficiency may be increased by increasing the stocking density to 200 larvae mL^{-1}, but further corroboration is required. Future studies could test higher initial stocking densities and lower algal residual background levels to increase efficiency. Along with the practical guidelines, the functional morphology descriptions for *P. zelandica* larvae may be used as a reference for hatchery operators to assess the health and performance of geoduck larvae, according to which daily actions may be adjusted.

"I cannot make anyone understand what is happening inside me. I cannot even explain it to myself"

- Franz Kafka, The metamorphosis

After becoming competent, bivalve larvae undergo a metamorphosis event in which the loss of larval-specific organs and emergence of juvenile-specific structures occur (Hadfield and Paul, 2001). The loss and emergence of some organs are taken place parallel to the reorientation and development of the remaining organs (Elston, 1999). Hence, substantial mortalities can occur at this time in both natural and hatchery conditions (Helm et al., 2004). Thus, larval metamorphic induction was tested with different neuroactive compounds: potassium ions, epinephrine hydrochloride, and acetylcholine chloride, which are commonly used in bivalve hatcheries, to improve geoduck spat yield (Chapter 6). It was unexpected that none of tested neuroactive compounds had positive effects on the metamorphic rate at either 3 or 24 h post-exposure. The negative results in these trials suggest that inducers used effectively in other bivalve species may not be transferrable to *P. zelandica*. Therefore, an approach based on gaining a better understanding of the likely triggers and metamorphosis process in *P. zelandica* may be appropriate. Further studies might incorporate 'omic' approaches to identify potential inducers, such as conspecific adults and bacterial biofilms, and to elucidate the mechanisms responsible for geoduck larval settlement and metamorphosis. Successful larval rearing and spat production is the license to establish a geoduck aquaculture industry.

"Any fool can know. The point is to understand."

Albert Einstein – a physicist

The main concerns of aquaculturists are survival and growth of animals within any culturing condition. However, understanding the underlying mechanisms driving growth and survival is as important as knowing the number of animals that survive or their growth rates. With ecophysiological knowledge of the animals, aquaculturists can optimize growth conditions as well as predict the animals' performance. Juvenile geoducks are typically field out-planted in either intertidal or subtidal areas when they reach 10-20 mmm size, and then the adults are harvested after 6-7 years. However, no research has been conducted to determine the effects of temperature and hypoxia on the growth or fitness of juveniles and adults, which are planted. This study is the first report on the effects of temperature and hypoxia on the aerobic performance ability of both juvenile and young adult geoducks (Chapter 8 and 9). Since it is well-known that small/young and large/old animals have different physiological rates, it is extremely important that those rates are standardized by size before any comparison between groups can be made. Hence, this study determined the allometric coefficients for geoducks' respiration rate (0.72) and clearance rate (0.63) (Chapter 7). These coefficients were used in Chapter 8 and 9 to standardize the physiological rates of juveniles and young adults and are recommended to be used in any future research on geoduck physiology. The optimal temperature for the aerobic scope of juvenile geoducks ranged between 11-15°C, while that for adults was 11-19°C. In addition, hypoxia had a significant effect on the aerobic scope of juvenile geoducks, but not adults. This quantitative information will improve the guidelines for farm site selection. It must be recognized that there is a practical problem in directly measuring the growth of juveniles and adults in short-term experiments, since geoducks are

slow-growing species. It takes months for juveniles and years for adults to show any difference in growth, and maintaining animals at different temperatures or under hypoxia for such a long time is not feasible in laboratory conditions. Over such a long period, the growth metric is confounded by the integration of seasonal factors in the field. Hence, it is difficult to determine the extent of temperature and hypoxia effects. Thus, the ecophysiological approach used in this study not only answers the basic concerns (i.e. growth and survival) in aquaculture, but also potentially provides underlying mechanism for the animal's performance. Hence, the present study illustrates how the ecophysiological approach can be usefully integrated into a practical aquaculture industry. Further research may investigate the effects of thermal acclimation at high temperatures on the aerobic scope of geoducks or the effects of hypoxia and hyperoxia on critical temperature levels.

10.3 Geoduck aquaculture vision as a conclusion

In 2011, the New Zealand aquaculture industry exported 1,667 ton of oysters, 5,166 tons of salmon, and 38,143 tons of mussels (AQNZ, 2012). These productions by value were NZ$298.1, of which NZ$16.6 million were from oyster (6%), NZ$63.4 million from salmon (21%), and NZ$218.1 million from mussels (73%) (AQNZ, 2012). The goal of the New Zealand aquaculture industry is set for NZD$1 billion of export values in 2025 (Carter, 2012). As a high value species, geoducks can contribute significantly to this goal. Assuming that 2 million juvenile geoducks will be produced, with 50% survival as the worst case scenario, 1 million geoducks will be harvested, which will be equivalent to 800 tons (0.8 kg each). If the landed price of geoducks is NZ$20 kg^{-1}, the landed value will be NZD$16 million. Subsequently, the export value will be

$NZD20 million, which results in meeting 4% of the goal. In the hatchery phase, to produce 2 million juvenile geoducks, it is assumed that the survival from pediveliger to juvenile is 10%, with the provided larval rearing practices and results in this study (100 larvae mL^{-1} and 70% survival), two 170 L tanks which are currently used to produce oyster and mussel larvae (Cawthron Institute, Nelson) can produce a sufficient number of pediveligers. Also, with the provided fertilization practices in this study (sperm:egg ratio of 50:1, > 90% normal embryo) 15 females, which release 2 million eggs each can produce a sufficient number of D-larvae. Although further research needs to be conducted to increase the survival from pediveliger to juvenile stage, these required hatchery resources for the scenario of 2 million juvenile geoduck production are applicable. Thus, it is feasible that geoduck can be the next big ticket item to achieve New Zealand's ambitious aquaculture export goals.

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